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Lactobacillus plantarum LBP-K10의 Cyclic dipeptides 와 cyclic dipeptide synthetase

Cyclic dipeptides and cyclic dipeptide synthetase of *Lactobacillus plantarum* LBP-K10

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Cyclic dipeptides and cyclic dipeptide synthetase of *Lactobacillus plantarum* LBP-K10

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ABSTRACT

Lactic acid bacteria (LAB) have been recognized to be a probiotic microorganism for human health. Cyclic dipeptides (CDPs) produced by LAB are important for their antibiotic activities. CDPs were a group of molecules with several kinds of diketopiperazine structure and have served as attractive scaffolds for drug design. However, the entire pool of CDPs from a given lactic acid bacterial culture is not fully understood. The present study identified the CDPs produced by *Lactobacillus plantarum* LBP-K10, which was the most potent antimicrobial isolates from Korean kimchi. Totally, seven CDPs were identified through gas chromatography-mass spectroscopy. Additionally this study provided a new method for purifying a massive amount of the complete set of CDPs from culture filtrates (CFs) of *Lb. plantarum* LBP-K10 by using the anion exchange resin Amberlite IRA-67 or Purolite A420S. The set of all CDPs showed remarkable potency with bioactivities against multidrug-resistant bacteria, pathogenic fungi and influenza virus compared with the activities of single CDP.

Recently, studies revealed lactic acid bacteria or its products might capable of inhibiting cancer progression by several mechanisms. However, most of studies focus on gastrointestinal (GI) tract related cancer, especially colon cancer. The effect of lactic acid bacteria or its related products on non-GI tract cancer was rarely examined. Since studies proved the effectively absorbing of CDPs, the cancer prevention effect lactic acid bacteria on a kind of non-GI tract cancer, breast cancer was investigated. A special

methylene chloride extracted fraction (MC-K10) was identified, which mainly contained several CDPs from *Lb. plantarum* LBP-K10 culture filtrates (CF-K10). In this study, it was firstly proved that oral applying CF-K10 inhibited the progression of breast cancer growth in animal model. We further discovered that CF-K10 and MC-K10 might induce apoptosis in MDA-MB-231 cells. Moreover, it was demonstrated MC-K10 and cyclo(Phe-Pro) can impair the stemness and self-renew of cancer stem cells. These studies provide a new insight for the probiotic actives of LAB and its products, as well as give a hint for developing breast cancer prevention strategy.

The importance and benefit of the CDPs on human heath encouraged us to investigate the cyclic dipeptide biosynthesis process. By ninhydrin activity staining, it was confirmed bioactive cyclic dipeptides synthesized in by *Lb. plantarum* LBP-K10 through cyclic dipeptide synthase (CDPS) dependent pathway. Then this CDPS were purified and characterized by 2D LC/MS analysis as a 26.1 kDa novel protein (accession number: gi311821850). The 690 bp gene sequence of this 26.1 kDa protein was also obtained from NCBI genome database. Interestingly, after transfected this gene into *E. coli*, the content of several proline base cyclic dipeptides was increased. When subjected to *in vitro* reaction conditions, CDPS helped to generate several cyclic dipeptides. To better understand that the mechanism of cyclic dipeptides synthesis in molecular level, X-ray analysis of CDPS was performed. CDPS was composed as a tetramer structure with 4 identical subunit showed in the ribbon model. This study for the first time

identified a novel CDPS and defined *in vitro* reaction conditions based on CDPs for generating CDPs. This study will help to understand cyclic dipeptides synthesis mechanism and producing nature antibiotic cyclic dipeptides *in vitro* through biosynthesis reactor.

Above of all, these studies identified the entire CDPs profile in MC extract fraction of *Lb. plantarum* LPB-K10, proved the cancer prevention effect of LAB and its produced CDPs, and for the first identified a novel CDPS. This study provided new insight on the probiotic LAB and benefit for understanding CDPs biosynthesis mechanisms.

Key words: Cyclic dipeptides; Cyclic dipeptide synthase; Antibiotic activity; *Lactobacillus plantarum*; Breast cancer; Cancer prevention; Cancer stem cells; Multidrug-resistant bacteria, Pathogenic fungi, Influenza A virus; Cyclo(Phe-Pro)

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LIST OF ABBREVIATIONS

bp	base pair
CDPs	cyclic dipeptides
CDPS	cyclic dipeptide synthetase
CI	chemical ionization
Da	dalton
DEAE	diethylaminoehanol electrophoresis
DKP	diketopiperazine
EI	electro ionization
GC	gas chromatography
H3N2	influenza A
HPLC	high performance (pressure) liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria-Bertani
MC	methylene chloride
MDCK	Madin Darby canine kidney
MIC	minimum inhibitory concentration
MRS	Rogosa and Sharpe
MS	mass spectrometry
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
PAGE	polyacrylamide gel
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfat

CHAPTER I.

Introduction

1. 1. General introduction of lactic acid bacteria and cyclic dipeptides

Lactic acid bacteria (LAB) are types of microorganisms that produce lactic acid as the primary and major metabolite when metabolizing sugar. Several type of LAB are widely known as probiotics since they are benefit for host animal by improving its intestinal microbial balance (Salminen *et al.* 1998). Actually, this group of probiotic bacteria are highly selected strains, including *Lactobacillus* spp., *Bifidobacterium* spp. *Streptococcus* spp. *et al,* which are capable of surviving in animal stomach (Conway *et al.* 1987) and defining gut survival properties and associated biological activates and which can be ingested in fermented milk products or as a supplement (Rafter 2002). Indeed, the probiotic effective of these kinds of strains are still accumulating and have been well reviewed (Salminen *et al.* 1998, Naidu *et al.* 1999, Masood *et al.* 2011). Beside their benefit for the nutrition and bowel activity, various disease prevention function of these probiotic strains were illustrated, especially relate to specific diseases, such as diarrhea (Chouraqui *et al.* 2004), lactose intolerance symptoms (Gorbach 1990), constipation (An *et al.* 2010), inflammatory bowel disease (del Carmen *et al.* 2010) as well as cancers (Adachi 1992, Hirayama and Rafter 2000, Rafter 2002).

Among the broad spectrum of probiotic activity of LAB, the antimicrobial activities were under intensive studies. LAB have been considered to have antagonistic effects, such as inhibition of adherence, establishment, replication and various pathogenic actions in intestinal and food-borne pathogens in bench studies (De Vuyst and Vandamme 1994). In many types of naturally fermented foods, which originate from animals and plants (Lindgren and Dobrogosz 1990, Holzapfel *et al.* 2001). Studies have

shown the fermented mixture containing *Lb. acidophilus* is inhibitory to *Shigella dysenteriae, Salmonella typhimurium,* and *Escherichia coli (*Rani and Khetarpaul *1998)* and inoculation of *Lb. reuteri* could suppresse *Cryptosporidium parvum* infection in C57BL/6 female mice (Alak *et al*. 1997). *Lactococcus lactis*, *Streptococcus cremoris* R3, *Lactococcus diacetylactis* V1and *S. thermophilus* T2 strains showed wide inhibitory spectra against the Gram-positive bacteria (Mezaini *et al.* 2009). For the antifungal ability, *Lb. acidophilus, Lb. acidophilus* reuteri, *Lb. casei* GG, and *Bifidobacterium animalis* were investigated by protecting athymic and euthymic mice from systemic candidiasis (Wagner *et al.* 1997, Gerez *et al.* 2009). *Lb. coryniformis*, *Lb. plantarum* and *Pediococcus pentosaceus* were also active against pathogenic fungi (Magnusson *et al.* 2003, Valerio *et al.* 2009). In this study we isolate a strain of LAB with strong antibiotic activity.

Most of the studies declared LAB can pass through the GI track alive and adherent to intestine wall. This alive adherent cells on intestine wall execute their probiotic active effect by secreting functional compounds during their proliferation. However, there are studies on antimicrobial indicated that these effective compounent may be found in culture filtrates (CFs) and these CFs may contribute for the benefit of human health.

LAB generate abundance metabolites that induce a broad range of influence on their host or themselves. For example, organic acids produce by LAB are responsible for the decrease in the pH, under anaerobic conditions, hydrogen peroxide induce the environmental redox potential (Ricke 2003). Antimicrobial small molecules, as secondary metabolites of LAB, have been investigated in relation to cellular metabolism

during cell growth or fermentation, such as, bacteriocin-like peptide, organic acid, cyclic dipeptides (CDPs) and other small chemical molecules (Niku-Paavola *et al.* 1999, Gänzle *et al.* 2000, Ström *et al.* 2002). Indeed, bacteriocin-like peptides, including bacteriocin, plantaricin and pediocin, have been demonstrated to be against Gram-positive and Gram-negative bacteria (Biswas *et al.* 1991, Abee *et al.* 1995) and short chain fatty acids inhibit microbe growth. Most importantly, Furthermore, cis-cyclo(L-Leu- L -Pro), cis-cyclo(L-Phe-L-Pro) and cis-cyclo(L-Val-L-Pro) were observed to be the most potent antiviral and antifungal CDPs from *Lb. plantarum* isolates (Gänzle *et al.* 2000). We have proved LBP-K10 CF contains at least of one functional cyclic dipeptide with antibiotic activities.

Cyclic dipeptides which consist of several kinds of diketopiperazines such as 2, 3-, 2, 5- and 2, 6-isomers (Kwak *et al.* 2013, Kwak *et al.* 2014), have been suggested to have broad range of biological effects. They may function as quorum sensing signal molecules through reducing virulence factor production and cross talk with other bacterial strains as well as various antibiotic functions (Holden *et al.* 1999, Campbell *et al.* 2009, Li *et al.* 2011). Cyclo(Phe-Phe) inhibited the serotonin transporter and acetylcholinesterase as a dual inhibitor *in vitro*, preventing the development of Alzheimer's disease and dementia (Tsuruoka *et al.* 2012). Indeed, cyclic dipeptides participate in many cellular signaling pathways, such as Cyclo(Arg-Pro) as chitinase inhibitor CI-4 from a marine *Pseudomonas* sp. IZ208 (Izumida *et al.* 1996), and cyclo(dehydroAla-Leu) as α-Glucosidase inhibitor from *Penicillium* sp. F70614 were identified, respectively (Kwon *et al.* 2000). Cyclo(Phe-Pro) and cyclo(Tyr-Pro) from *Lb.*

reuteri were identified as signaling effectors in quorum sensing by attenuating virulence factor produced by *staphylococci* (Li *et al.* 2011). Interestingly, anti-hyperglycemic activity of zinc plus cyclo(His-Pro) suggested that oral intake of an optimal usage of cyclic dipeptides could control blood glucose concentrations by stimulating muscle glucose consumption in rats (Song *et al.* 2003). Recently, it was demonstrated that *in vitro* assessment regarding the probiotic and functional potential of Lactobacillus strains displayed the antagonistic activity against several types of pathogens along with the depletion of cholesterol and nitrate, free radical scavenging, immune response stimulation and highly production of exopolysaccharides (Ren *et al.* 2014). Additionally, *Lactococcus* spp., and *Pediococcus* spp. strains isolated Kefir also have a metabolic activity significantly similar to *Lactobacillus* spp. when observing the potential of probiotic properties (Sabir *et al.* 2010). In this study, we focused on these CDPs compounds generated by LAB.

1. 2. Discovering antimicrobial CDPs in LAB

The secondary metabolites produced by LAB have been investigated for small compounds with antimicrobial activity (Naidu *et al.* 1999, Rouse and van Sinderen 2008). Of the different LAB strains, *Lactobacillus* spp. are known to produce low molecular weight antibiotic substances during metabolic processes (Messens and De Vuyst 2002) Among antibiotic small compounds from LAB culture filtrates, various different types of CDPs, which consist of several kinds of diketopiperazines such as 2, 3-, 2, 5- and 2, 6-isomers (Kwak *et al.* 2013, Kwak *et al.* 2014), Considering the

antimicrobial compounds from *Lactobacillus* CFs, different types of cyclic dipeptides (CDPs), which consist of several kinds of diketopiperazines, including the 2, 3-, 2, 5- and 2, 6-diketopiperazines, have been widely investigated due to the potent antimicrobial functions against Gram-positive, Gram-negative bacteria and fungi (Witiak and Wei 1990, Prasad 1995, Dinsmore and Beshore 2002).

This diketopiperazines-ring system is formed through the condensation of two amino acids resulting in the smallest possible cyclic peptide (Eguchi and Kakuta 1974, Prasad 1995, Bettens *et al.* 2000).

Cyclic dipeptides have received an increasing attention since the first report in 1924 (Abderhalden and Komm 1924), because of their diverse and bioactive activities, such as antitumor (Kanoh *et al.* 1999, Kanzaki *et al.* 2000), antiviral (Sinha *et al.* 2004) , antifungal (Asano 2003) (Houston *et al.* 2004), antibacterial (Sugie *et al.* 2001, Fdhila *et al.* 2003), antiprion, antihyperglycemic or glycosidase inhibitor activities as well reviewed by P De Carvalho, M. *etc.* (P de Carvalho and Abraham 2012). Cyclo(Trp-Trp) from *Streptomyces* sp. KH29 showed significant inhibitory activity against multidrug-resistant *Acinetobacter baumannii* and also other bacterial and fungal strains (Lee *et al.* 2010).

Antibacterial synergistic activity of cyclo(Phe-Pro) and cyclo(Leu-Pro) from *Streptomyces* species has been reported against bacteria, even vancomycin-resistant *Enterococcus faecium, Candida albicans* and *Cryptococcus neoformans* (Rhee 2004).

Cyclo(Pro-Trp), cyclo(Phe-Pro), cyclo(Trp-Pro), cyclo(Trp-Trp), and histidine-containing cyclic dipeptides were inhibitory to fungal strains such as *C. albicans,*

Aspergillus niger and *Penicillium notatum* (Graz *et al.* 1999, McCleland *et al.* 2004). Cyclo(Phe-Pro) and cyclo(Ile-Pro) from cell-free filtrates from propionibacteria also showed an antifungal activity against *As. fumigatus* and *Rhodotorula mucilaginosa* (Lind *et al.* 2007). Cyclo(Leu-Pro) and cyclo(Phe-Pro) from *Lb. plantarum* FST 1.7, *Streptomyces* sp. KH-614, *Achromobacter xylosoxidans,* and *Lb. plantarum Mi.* Lactic acid bacteria 393 have been responsible for antimicrobial activity against bacteria and fungi (Ström *et al.* 2002, Yan *et al.* 2004, Dal Bello *et al.* 2007).

Recently, we also demonstrated the bioactivity of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe- L-Pro) against influenza A virus (Kwak *et al.* 2013), along with *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) inhibitory to the growth of plant and human pathogenic *Ganoderma boninense* and *C. albicans*, respectively (Kwak *et al.* 2014). In this study, we for the first time identified the entire compounds of CDPs pool in MC extracted fraction produce by *Lb. plantarum* LBP-K10 and confirmed their antibiotic effect.

1. 3. Cancer prevention effect of LAB and its metabolic CDPs

As mentioned above, the cancer prevention effect of CDPs has been discovered. Glycine-containing cyclic dipeptides, cyclo(Phe-Cys), and especially cyclo(Tyr-Cys) significantly inhibited cancer cell growth in HT-29, HeLa, and MCF-7 cells *in vitro* (Van der Merwe *et al.* 2008). Moreover, synthetic CDPs, such as cyclo(His–Phe) and cyclo(His-Tyr), have been reported to modify cellular ion channels, thereby inducing cell death in HeLa (cervical), WHCO3 (oesophageal) and MCF-7 (breast) carcinoma

cell lines and inhibiting the proliferation of bacteria and fungi (McCleland *et al.* 2004, Yan *et al.* 2004). Previously, we also successfully separated and purified a group of small molecules from a special strain of *Lb. plantarum* LBP-K10, with significant anti-microorganism effect. These small molecules, named cyclic dipeptides (CDPs), are consist of several kinds of diketopiperazines such as 2, 3.-, 2, 5 and 2, 6-isomers (Kwak *et al.* 2013, Kwak *et al.* 2014). Including our studies, the existence of CDPs in LAB culture supernatant have been widely confirmed (Li *et al.* 2011, Perzborn *et al.* 2013). Besides the anti-microorganism activity, the tumor prevention effects of CDPs are also described. CDPs are found capable of inducing cancer cell apoptosis (Brauns *et al.* 2004, Brauns *et al.* 2005) or preventing gene mutation (Rhee 2004).

However, most of anticancer studies are focused on gastrointestinal cancer cells, mainly on colon cancer and executed *in vitro* cell lines. Since the anti-cancer effect of CDPs was well demonstrated as above and studies confirmed the rapid absorption of CDPs in animal (Mizuma *et al.* 1998), we were encouraged to investigate of the cancer prevention effect of the specific LAB stain, *Lb. plantarum* LBP-K10, recent isolated in our team, which generating serial functional CDPs, on non-gastrointestinal cancer model**.** In this study, we discovered that oral in taking heat inactive cell-free of *Lb. plantarum* LBP-K10 (CF-K10) might effectively prohibit the progression in situ breast cancer progression in mouse transplantation model possibly through apoptosis pathways induced by CDPs.

1. 4. Cyclic dipeptides synthesis and cyclic dipeptide synthetase

Since CDPs have these remarkable effect on anti-microorganism and even on cancer prevention, synthesis of this kind of molecules were intensively studied. Studies on the cyclic dipeptides have been focused on structures and synthetic methods related to their antimicrobial functions (Prasad 1995, O'Neill and Blackwell 2007, Trabocchi *et al.* 2008, Budesinsky *et al.* 2010). The molecular scaffolds in cyclic dipeptides have been recognized as a privileged structural in the search for synthetic or semisynthetic pharmacological compounds (Prasad 1995, Izumida *et al.* 1996). Synthetic cyclic dipeptides such as cyclo(His–Phe) and cyclo(His-Tyr) were reported to modify cellular ion channels, thereby induce cell death in HeLa (cervical), WHCO3 (oesophageal) and MCF-7 breast carcinoma cell lines and furthermore inhibit proliferation of bacteria and fungi (Menard *et al.* 2004). And also, an albonoursin-producing strain, *Streptomyces albulus* KO-23 was reported to catalyze the conversion of cyclic dipeptides into the corresponding dehydro derivatives, which were observed to interfere with cell division (Kanzaki *et al.* 2000). A large number of diketopiperazines natural products could be obtained by chemical synthesizing route (Witiak and Wei 1990, Dinsmore and Beshore 2002), which, however, generally are inefficiency and high-cost.

Besides the chemical synthesizing route (Delaforge *et al.* 2001, Horton *et al.* 2002, Wang *et al.* 2002), cyclic dipeptides have been isolated from microbial sources or various foods. These evidences indicated the existance of biosynthesis route for cyclic dipeptide molecules (Jayatilake *et al.* 1996, Magnusson *et al.* 2003, Schnürer and Magnusson 2005). It has long been known that nonribosomal peptide synthetases are able to generate diketopiperazines-containing compounds, either through a dedicated

pathway or through the premature release of peptidyl intermediates via cyclization (Dal Bello *et al.* 2007, Giessen *et al.* 2013). The discovery of the albonoursin biosynthetic gene cluster in *Streptomyces noursei* and the characterization of the first cyclic dipeptide synthase AlbC (Belin *et al.* 2012, Li 2012), make it possible to form two successive peptide bonds in an ATP-independent fashion by hijacking aminoacyl-tRNAs from the ribosomal machinery (Belin *et al.* 2012). The *Mycobacterium tuberculosis* CDPS was found to generate cyclodityrosine through the formation of an aminoacyl-enzyme intermediate using aminoacyl-tRNAs as substrates (Bonnefond *et al.* 2011). Actually, cyclic dipeptides can directly using aa-tRNAs as substrates for the generation of a diketopiperazines-ring system (Yan *et al.* 2004). However, the knowledge of cyclic dipeptide synthesis still limited until now. In this study, we identified a novel CDPS from LAB and for the first time confirmed it capable of catalyzing amino acid to produce CDPs molecules *in vitro* and *vivo.*

1. 5. Contents of this study

1. 5. 1. Profiling entire CDPs compounds in *Lb. plantarum* LBP-K10

Although the intra- and extracellular functions and the bioactivity of single CDPs have been revealed steadily for decades, all of the components of the CDPs pool, which is naturally produced by LAB, regarding the bioactivity or bio-significance in a given LAB culture have not been clearly identified. Herein, eight types of CDPs and one non-peptidyl antibiotic component were isolated by high-performance liquid chromatography (HPLC) and confirmed by gas chromatography-mass spectroscopy

(GC-MS). On the basis of our previous studies regarding the antimicrobial and antiviral functions of a single CDP (Kwak *et al.* 2013, Kwak *et al.* 2014), we herein propose a method for purifying a massive amount of the total pool of CDPs, designated K10-CCDP, from *Lb. plantarum* LBP-K10 CFs. We tried to address the significant bioactivity of both single CDPs and K10-CCDP, which was obtained from culture filtrates after removing impurities including organic acids or sugars through anion exchange chromatography (AEC). Thus, we identified a set of CDPs with antibiotic activity from a specific LAB species and provide a strategy for characterising the total set of CDPs from LAB cultures for the first time.

1. 5. 2. CDPs participated in cancer prevention effect of LAB on breast cancer

Although many studies investigated the anticancer activities of LAB, its effect on non-GI tract cancer has been rarely studied. Since the absorption of CDPs was proved previously and the stability of its special chemical structure, we expect LAB are capable of inhibiting the growth of breast cancer. In this study, the cancer prevention effect of LAB were confirmed first on in situ breast cancer model in mice and further revealed LAB may induced apoptosis in breast cancer cell line MDA-MB-231. Moreover, the growth inhibition effect of MC-K10 and cyclo(Phe-Pro)on cancer stem cell were also observed. These results provided new insight for the probiotic active of LAB, and also contribute to develop new strategies for breast cancer privation.

1. 5. 3. Identifying a novel CDPS in *Lb. plantarum* LBP-K10

It was interested in identifying the CDPS, which synthesized the functional cyclic dipeptides. In this study, CDPS-like function of a novel protein (gi311821850) were reported, then the bioactive cyclic dipeptides of production, which in *Lb. plantarum* LBP-K10 through cyclic dipeptides synthase dependent pathway, were confirmed by ninhydrin activity staining. And the *in vitro* cyclic dipeptides synthesis conditions, based on the newly found CDPS protein, was successfully developed. After transfected this gene into *E. coli*, the expression of several identifying L-Proline base cyclic dipeptides were increased. The production of cyclic dipeptides were confirmed by high performance liquid chromatography and confirmed by gas chromatography-mass spectroscopy. The synthesis of cyclic dipeptides could be observed by HPLC when CDPS was applied *in vitro* conditions. To confirm the peaks in high-performance liquid chromatography represented cyclic dipeptides, each compound according each peak were collected and subjected to gas chromatography-mass spectroscopy analysis. To better understand that the mechanism of cyclic dipeptide synthesis in molecular level, X-ray analysis of CDPS was performed. This study elaborated the mechanism of cyclic dipeptides synthesis in lactic acid bacteria, and provided an opportunity for producing antibiotic cyclic dipeptides *in vitro* synthesis and its physiological roles need to be fully investigated.

CHAPTER II.

Identification of cyclic dipeptides produced by *Lactobacillus plantarum* LBP-K10

2. 1. Aims

- **Profiling of entire CDPs compounds in MC extract of CF-K10**
- **Structure identifying of each CDPs compounds**
- **Antibiotic effect of each CDPs in MC extract of CF-K10**
- **Antibiotic effect of CDPs complex conditioned by *Lb. plantarum* LBP-K10**

2. 2. Materials and methods

2. 2.1. Strains

All bacterial, fungal and viral strains were listed in Table 1. *Lb. plantarum* LBP-K10 and other isolates from Korean fermented kimchi were identified by 16S rDNA sequencing method and were cultured in mMRS as previously reported (Kwak *et al.* 2013). Serially diluted bacterial cells were plated and grown on mMRS for CFU (ml) enumeration during cell growth (Panagou *et al.* 2007).

All experiments were performed by using each single compound or K10-CCDP, which correspond to three different sets of CDPs from the CFs without organic acids, without sugars, or without organic acids and sugars, respectively.

Antibacterial activity against multidrug-resistant bacteria and reference strains was measured every 24 h after seed inoculation and dilution method was used to determine the minimum inhibitory concentration (MIC) of antimicrobial substances (Huys *et al.* 2002, Paulo *et al.* 2010).

G. boninense isolate (GMR3) and *C. albicans* were grown and maintained to evaluate antifungal activity as described previously (Kwak *et al.* 2014).

A plaque assay for antiviral activity was performed using Madin-Darby canine kidney (MDCK) cells grown in complete DMEM, and influenza A(H3N2)virus for infection as proposed previously (Kwak *et al.* 2013)

Table 1|Strains, primers, and plasmids used in this study

Strain	Types or strains	Source or reference
LAB strains		
Ln. kimchii LBP-B02	Original isolate from fermented mustard leaves and stems	This study
W. cibaria LBP-B06	Original isolate from fermented mustard leaves and stems	This study
Lb. sakei LBP-S01	Original isolate from fermented stonecrop	This study
Lb. plantarum/pentos LBP-S02	Original isolate from fermented stonecrop	This study
Lc. lactis LBP-S03	Original isolate from fermented stonecrop	This study
S. sciuri LBP-S07	Original isolate from fermented stonecrop	This study
Ln mesenteroides LBP-K06	Original isolate from fermented Chinese cabbage	This study
Lb. plantarum LBP-K10	Original isolate from fermented Chinese cabbage	Kwak *et al*., 2013
Ln. citreum LBP-K11	Original isolate from fermented Chinese cabbage	This study
W. cibaria LBP-K15	Original isolate from fermented Chinese cabbage	This study
W. confusa LBP-K16	Original isolate from fermented Chinese cabbage	This study

Multidrug-resistant bacteria		
Gram-positive bacteria		
S. aureus 11471	oxacillin-resistant *S. aureus* (ORSA) 11471, which is resistant to beta-lactam antibiotics, including penicillins (methicillin, dicloxacillin, nafcillin and oxacillin) and cephalosporins	This study, KNIH [a]
S. pneumoniae 14596	*Streptococcus pneumoniae* 14596, which is resistant to penicillin, erythromycin, tetracycline and clindamycin	This study, KNIH [a]
Gram-negative bacteria		
S. typhimurium 12219	*Salmonella typhimurium* 12219, which is resistant to ACSSuT (ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline)	This study, KNIH [a]
Pathogenic fungi		
C. albicans SC5314	Wild type isolate	Fonzi and Irwin, 1993
G. boninense	GMR3,wild type isolate	This study

This was supported by Korea National Institute of Health

2. 2. 2. Culture filtrate fractionation

The supernatant from three-day cultured *Lb. plantarum* LBP K-10 was collected as previously described (Kwak *et al.* 2013). To obtain each CDP or K10-CCDP, filtered samples were separated using a semi-preparative HPLC system (Agilent, USA) with a semi-preparative Hypersil ODS C18 reverse-phase column (9.4 × 250 mm, Agilent, USA) as proposed previously (Kwak *et al.* 2013).

2. 2. 3. Mass analysis and X-ray crystallography

To perform electron ionization (EI) and chemical ionization (CI) of each fraction, GC-MS (Agilent, Germany) was utilized (Kwak *et al.* 2013). A chromatographic system consisting of an Agilent 6890 series GC equipped with a 7679 series automatic liquid sampler was used. Mass analysis was conducted using a high-resolution mass spectrometer (JEOL JMS-700, Japan).

Crystals of bioactive CDPs were coated with paratone-N oil, and the diffraction data were measured at 95 K with synchrotron radiation (λ = 0.66999 Å) on an ADSC Quantum-210 detector with a silicon (111) double crystal monochromator (DCM) at the 2D SMC beamline at Pohang Accelerator Laboratory, Korea (Kwak *et al.* 2013). These crystal structures were deposited at the Cambridge Crystallographic Data Centre (https://www.ccdc.cam.ac.uk/services/structure_deposit/).

2. 2. 4. K10-CCDP preparation

K10-CCDP was prepared from CFs using both primary AECs and a secondary immediate compound-refinement process. The secondary refinement procedure included the following three sequential steps: MC extraction of the eluents from the primary AECs, HPLC fractionation/collection only from F1 to F17 of this MC extract, and finally repetition of the MC extraction on each of the collected fractions.

Basically, we tried to remove organic acids and sugars from the CFs via three sets of AECs. To eliminate impurity-based side reactions and interference from organic acids or sugars, the resins Amberlite IRA-67 (Sigma-Aldrich, USA) and Purolite A420S (Purolite, USA) were selected as the weakly or strongly basic anion exchangers, respectively, with modifications (Maciel de Mancilha and Karim 2003, Moldes *et al.* 2003, Roja *et al.* 2005). Before the AEC was used to recover organic acids and sugars, Amberlite IRA-67 and Purolite A420S were converted into the free base OH^- or Cl^- forms, respectively. The CF was applied to Amberlite IRA-67 resin-packed open columns at a flow rate of 15 ml/min, and the unbound eluent was collected. Then, Purolite A420S was utilized to remove sugars from both the CFs and the organic acid-removed primary eluents. Additionally, the refining process was performed by extraction of the AEC eluents with 10-fold volumes of MC for further HPLC fractionation. To avoid collecting impurities, such as compounds that competitively bind to a C18 column during HPLC, the filtered samples were re-separated, and only seventeen fractions were collected. The resulting samples were again lyophilized, extracted with MC and filtered for antibiotic assays. Three types of K10-CCDP were prepared in this way and were designated according to the type of AEC that was used: K10-CCDP-I (CF without

organic acids), K10-CCDP-II (CF without sugars) and K10-CCDP-III (CF without organic acids and sugars) (Fig. 1).

2. 2. 5. Statistical analysis

The results are presented as the means ± standard deviation (SD). The statistical significance of the differences was tested using Student's t-test in Microsoft Office Excel (2013). For all comparisons, values of $p < 0.05$ (*) were considered statistically significant.

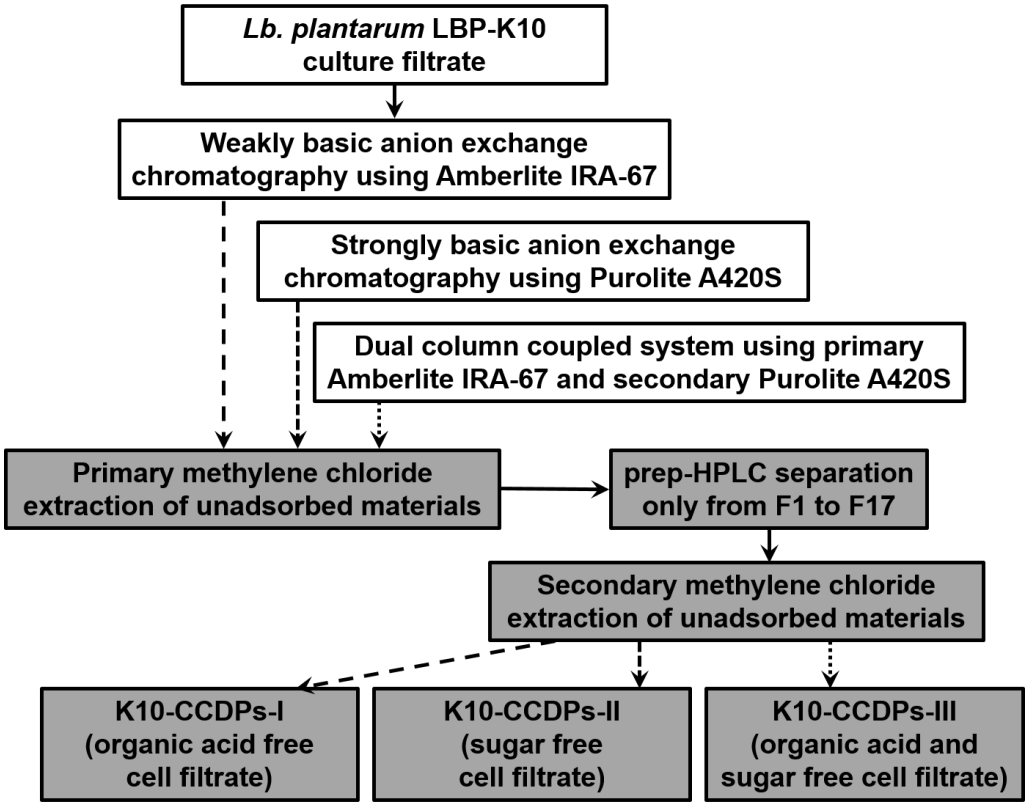


Fig. 1. Three types of K10-CCDP was prepared using various types of column chromatography followed by MC extraction. All procedures for purifying K10-CCDP from the *Lactobacillus* CFs are shown. The corresponding chromatography using weakly or strongly basic anion exchange is indicated (arrow).

2. 3. Results

2. 3. 1. Characterization of the potent antimicrobial isolate

We isolated approximately 400 strains from three types of Korean traditional kimchi and 200 strains were identified as LAB with an antimicrobial activity among these isolates (Table 2). Of these bacterial CFs, thirty isolates with potent antibacterial activity were selected and herein *Lb. plantarum* LBP-K10 CF exhibited the most significant activity against reference strains (Table 3 and Fig. 2A). These results consistence to studies on the *Lb. plantarum* strains originated from different varieties of kimchi, which showed a higher antimicrobial activity compared to any other isolate (Lee *et al.* 2007, Lim and Im 2009, Yang *et al.* 2011). Moreover, *Lb. plantarum* WCFS1 was suggested to be capable of encoding pyruvate-dissipating potential and being known as a fermenter with superb nutritional and bioactive properties (Kleerebezem *et al.* 2003). It was also demonstrated that *in vitro* assessment regarding the probiotic and functional potential of *Lactobacillus* strains displayed the antagonistic activity against several types of pathogens along with other physiological functions including the depletion of cholesterol and nitrate, free radical scavenging, immune response stimulation and highly production of exopolysaccharides (Ren *et al.* 2014). Therefore, to optimize the experimental conditions for the purification of antimicrobial substances in the isolated *Lb. plantarum* LBP-K10 CF, we preferentially observed the cell growth associated with antibacterial activity as proposed (Nardi *et al.* 2005). Despite the increased optical density, the CFU significantly inversely decreased after 32 h (Fig. 2B), at which time the pH

concomitantly decreased approximately 3.8 (Fig. 2C). This result indicates that changes in the CFU and pH values, which were both followed by the enhancement of antibacterial activity, might have been affected by metabolic by-products throughout the entire period (Fig. 2D). This preliminary evidence for the isolated bacterial CFs coincided with the bactericidal activity of CFs in the *Lb. plantarum* C-11, *Lb. sake* 706 and *Lb. murinus* strain L1 isolated from cucumber, meat and faeces of a Wistar rat, respectively (Schillinger and Lücke 1989, Nardi *et al.* 2005). Simultaneously, the amount of each fraction significantly increased during cell growth, which showed the time point with the highest total amount was 72 h (Fig. 2E). Based on these findings, we compared the production of antifungal and antiviral CDPs produced by *Lb. plantarum* LBP-K10 at 72 h (Fig. 3A) with reference to previously identified several types of L-proline based CDPs (Kwak *et al.* 2013, Kwak *et al.* 2014). Interestingly, all fractions of the isolates showed an identical fractionation pattern, although the relative amount in the fractions of the *Lb. plantarum* LBP-K10 CF was significantly higher than that in any other isolate (Fig. 3A). The fractions of CF from *P. pentosaceus*, which have a low amount of fractions compared to *Lb. plantarum* LBP-K10 and *Leuconostoc mesenteroides* LBP-K06, was shown, along with mMRS broth as a reference experiment (Fig. 4). This result suggests that despite the differences in the amount of substances among the isolates, LAB might synthesize and similarly excrete analogous metabolites functioning as bioactive mediators including antifungal and antiviral CDPs (Kwak *et al.* 2013, Kwak *et al.* 2014). Moreover, to verify the principal CDPs produced in the LAB, the three-day cultured *Lb. plantarum* LBP-K10 was harvested and regrown in 25 mM sodium phosphate buffer (pH

7.4) in the presence or absence of D-glucose (Fig. 3B). The significant higher amounts of the CDPs ranging from F11 to F15 were produced by L-proline supplemented *Lactobacillus* cells, particularly in the presence of D-glucose although the glucose-depleted buffer conditions did not cause a change in the growth rate (Fig. 3B). Based on the result of a significantly increased *cis*-cyclo(L-Leu-L-Pro) under all experimental conditions, this unique finding indicates that *Lb. plantarum* LBP-K10 might produce *cis*-cyclo(L-Leu-L-Pro) predominantly prior to other metabolites (Fig. 3B). Thus, due to the CDP-rich CF of *Lb. plantarum* LBP-K10 bearing the most potent antibiotic properties, this strain was used to purify all of the single CDPs and K10-CCDP in this study.

Table 2 LAB strains isolated from traditional korean fermented plant materials

	Source and strain number		
	Mustard leaves and stems	Stonecrop	Chinese cabbage
Leuconostoc spp.	93	10	28
Lactobacillus spp.	14	8	17
Lactococcus spp.	-	1	-
Weissella spp.	2	14	18

Table 3 The bioactivities of the CF from isolated LAB

Source	Strain	Antagonism test[a]*	MIC[b,c]*	Taxon confirmed by sequencing
Mustard leaves and stems	LBP-B01	++	+++	*Lb. sakei*
	LBP-B02	++	++	*L. kimchii*
	LBP-B03	++	++	*Ln. mesenteroides*
	LBP-B04	++	++	*Ln. mesenteroides*
	LBP-B05	+++	++	*Ln. paramesenteroides*
	LBP-B06	+++	++	*W. cibaria*
Stonecrop	LBP-S01	+ +	+++	*Lb. sakei*
	LBP-S02	+++	+++	*Lb. plantarum/pentos*
	LBP-S03	++	++	*Lc. lactis*
	LBP-S04	++	++	*L. citreum*
	LBP-S05	++	++	*L. citreum*
	LBP-S06	+	++	*L. lactis*
	LBP-S08	++	++	*W. hellenica*
Chinese cabbage	LBP-K01	++	+++	*Lb. plantarum*
	LBP-K03	++	++	*L. citreum*
	LBP-K04	++	–	*L. citreum*
	LBP-K05	++	++	*L. holzapfelii*
	LBP-K06	+++	++	*Ln. mesenteroides*
	LBP-K07	++	++	*Ln. pseudomesenteroides*
	LBP-K08	++	+	*Ln. mesenteroides*
	LBP-K09	++	++	*Lb. brevis*
	LBP-K10	+++	+++	*Lb. plantarum*

LBP-K11	++	++	*Ln. citreum*
LBP-K12	+++	++	*Ln. mesenteroides*
LBP-K13	++	++	*Ln. mesenteroides*
LBP-K14	++	++	*Ln. pseudomesenteroides*
LBP-K15	+++	++	*W. cibaria*
LBP-K16	++	++	*W. confusa*

[a] Symbol: +, <15 mm; ++, <22 mm; +++, >22 mm (Indicator strain: *B. subtilis)*

[b] MIC: Minimum inhibitory concentration

[c] Symbol: +, 1-fold; ++, 0.5-fold; +++, less than 0.25-fold (Indicator strain: *B. subtilis*)

* All experiments represent the average of three independent experiments.

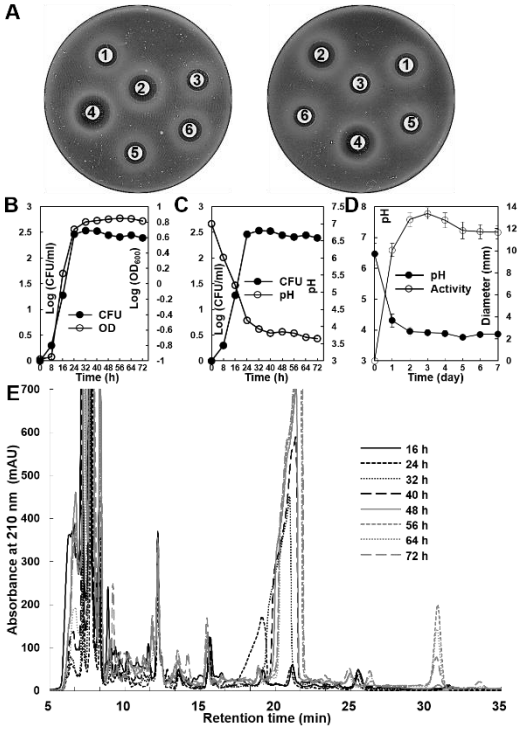


Fig. 2. The highest level of antimicrobial activity of *Lb. plantarum* LBP-K10 CF. (A) A comparison of the antibacterial activity of the following isolated lactic acid bacteria: (1) *Weissella cibaria* LBP-B06, (2) *Lb. sakei* LBP-S01, (3) *Ln. kimchi* LBP-B02, (4) *Lb. plantarum* LBP-K10, (5) *Ln. citreum* LBP-K11, and (6) *Ln mesenteroides* LBP-K06. Disc diffusion assays were performed using *B. subtilis* (left) and *E. coli* (right). (B) Cell growth, as indicated by the absorbance at 600 nm, and the corresponding cell viability as determined by counting the cell colony numbers (CFU ml^{-1}). (C) Decrease in pH during the growth of *Lb. plantarum* LBP-K10. (D) Antibacterial activity and pH of the *Lb. plantarum* LBP-K10 CF. (E) A significant increase in the antimicrobial and antiviral CDPs produced by *Lb. plantarum* LBP-K10 with respect to incubation time. The measured data values are representative of the average (± standard deviation) from three independent experiments.

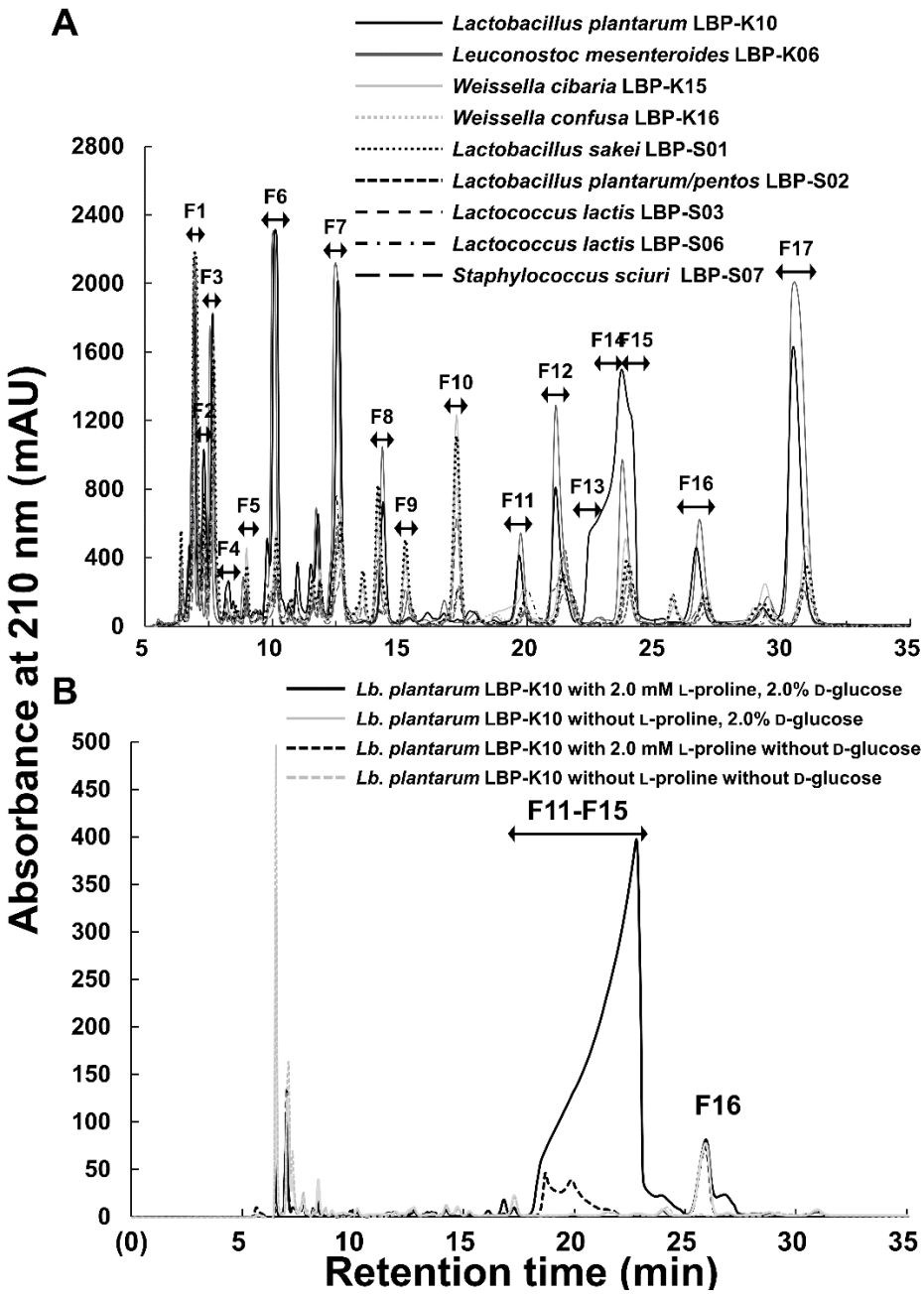

A
Lactobacillus plantarum LBP-K10
Leuconostoc mesenteroides LBP-K06
Weissella cibaria LBP-K15
Weissella confusa LBP-K16
Lactobacillus sakei LBP-S01
Lactobacillus plantarum/pentos LBP-S02
Lactococcus lactis LBP-S03
Lactococcus lactis LBP-S06
Staphylococcus sciuri LBP-S07
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
F13
F14
F15
F16
F17
Absorbance at 210 nm (mAU)
B
Lb. plantarum LBP-K10 with 2.0 mM L-proline, 2.0% D-glucose
Lb. plantarum LBP-K10 without L-proline, 2.0% D-glucose
Lb. plantarum LBP-K10 with 2.0 mM L-proline without D-glucose
Lb. plantarum LBP-K10 without L-proline without D-glucose
F11-F15
F16
Retention time (min)

Fig. 3. The production of a large amount of CDPs by *Lb. plantarum* LBP-K10 compared with various tested isolates.

(A) HPLC profiles of *Lb. plantarum* LBP-K10 and the following other isolated lactic acid bacteria: *Lb. sakei* LBP-S01, *Lb.plantarum/pentos* LBP-S02, *Lb.*LBP-S03, *Lb. lactis* LBP-S06, *Staphylococcus sciuri* LBP-S07, *Ln. mesenteroides* LBP-K06, *Lb. plantarum* LBP-K10, *W. cibaria* LBP-K15 and *Weissella confusa* LBP-K16. A chromatographic analysis of *Lb. plantarum* LBP-K10 was performed every 8 h with detection at 210 nm. The predominant production of *cis*-cyclo(L-Leu-L-Pro) by *Lb. plantarum* LBP-K10 under several conditions. (A) Changes in HPLC profiles of *cis*-cyclo(L-Leu-L-Pro). *Lactobacillus* cells were grown as described in the Experimental procedures after supplementation with 2.0 mM L-proline in mMRS. The filtrate from a three-day culture of *Lb. plantarum* LBP K-10 was extracted using methylene chloride for chromatographic analysis. (B) Changes in the biosynthesis of *cis*-cyclo(L-Leu-L-Pro). All grown *Lactobacillus* cells were harvested and washed twice. All experiments were performed in 25 mM sodium phosphate (Na_2HPO_4) buffer at a pH of 7.2, with or without 2 % glucose and with or without 2.0 mM L-proline.

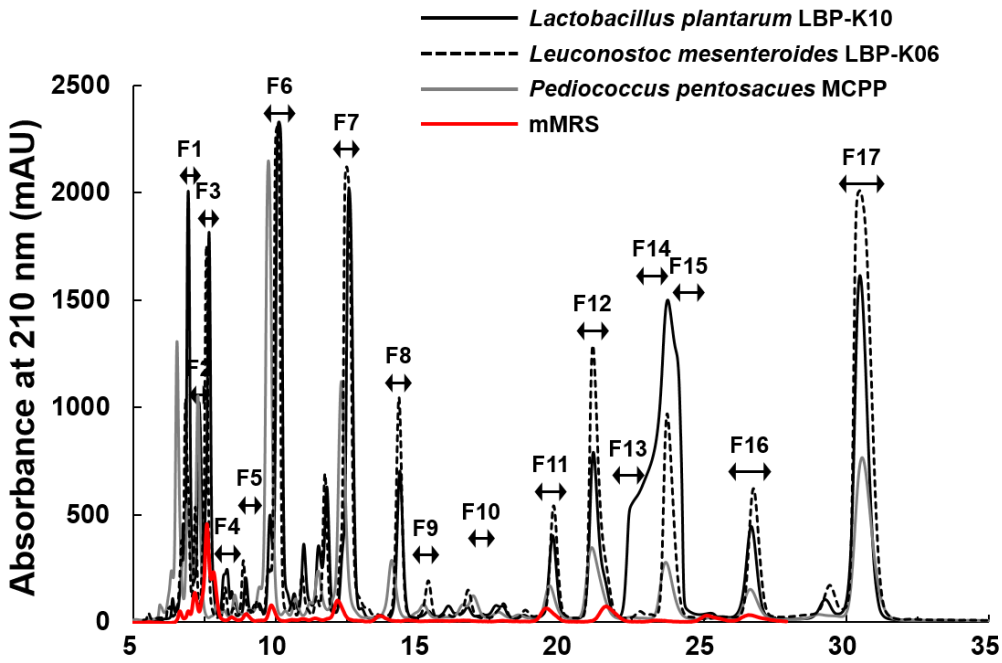


Fig. 4. HPLC fractionation pattern of the CFs in isolated lactic acid bacteria. A chromatographic analysis of the CFs driven by LAB strains was performed at 72 h with a wavelength at 210 nm. All experiments were performed at least three times independently.

2. 3. 2. Seventeen fractions from the CF contain sixteen CDPs

From three-day cultured *Lb. plantarum* LBP-K10 filtrate containing bioactive CDPs, seventeen fractions were isolated and characterized by GC-MS analysis or X-ray crystallography (Fig. 5). The fragmentation patterns in the EI-MS spectra of a series of corresponding fractions were completely identical to the CDPs that were characterized from various sources of food materials as proposed previously (Stark and Hofmann 2005, Wang *et al.* 2010). Surprisingly, the EI and CI values corresponding to all fractions were observed to be entire CDPs except for one fraction, including seven types of proline-based CDPs, one type of non-proline-based cyclo(Phe-Ala) and non-peptidyl DL-3-phenyllactic acid, respectively (Figs. 6, 7 and Table 4). Additionally, we previously reported several types of *cis*-configured diketopiperazines (Kwak *et al.* 2013, Kwak *et al.* 2014), partly including F7, F8, F13 and F17, and DL-3-phenyllactic acid. The crystal structures of active fractions have been assigned to the following deposition numbers: F7 (*cis*-cyclo(L-Val-L-Pro)-CCDC 937497) (Kwak *et al.* 2014); F8 (*cis*-cyclo(L-Leu-L-Hyp)-CCDC 937498); F13 (*cis*-cyclo(L-Leu-L-Pro)-CCDC 937533) (Kwak *et al.* 2013); F15 (DL-3-phenyllactic acid- CCDC 951328); and F17 (*cis*-cyclo(L-Phe-L-Pro)-CCDC 937534) (Kwak *et al.* 2013).

Table 4 Mass analysis of *Lb. plantarum* LBP-K10 fractions

Fractions	m/z of $[M+1]^+$	m/z (%) of EI-MS	Predicted molecules
F1 (6.5-7.0)[a]	261.0	56.1 (28.9), 61.2 (12.2), 70.2 (80.1), 76.2 (16.2), 85.2 (100.0), 86.2 (31.9), 91.2 (84.2), 98.2 (16.1), 99.2 (7.8), 111.3 (38.7), 128.3 (45.5), 141.3 (8.8), 148.3 (21.3), 154.3 (45.9), 155.3 (10.5), 170.4 (35.3), 184.4 (11.3), 196.4 (12.5), 244.5 (5.4)	cyclo(Tyr-Pro), $C_{14}H_{16}N_2O_3$
F2 (7.0-7.5)[a]	185.0	55.1 (18.7), 69.2 (30.2), 70.2 (44.4), 74.2 (2.6), 83.2 (52.7), 98.2 (20.4), 111.2 (75.9), 126.3 (8.7), 154.3 (100.0), 155.3 (11.1), 184.4 (1.3)	cyclo(Ser-Pro), $C_8H_{12}N_2O_3$
F3 (7.5-7.8)[a]	211.0	55.2 (17.8), 69.3 (28.6), 70.3 (68.3), 83.3 (41.7), 86.3 (10.6), 98.3 (17.0), 111.3 (57.3), 114.3 (3.7), 126.4 (9.1), 140.4 (2.3), 154.4 (100), 155.4 (10.9), 168.5 (9.8), 210.6 (17.6)	cyclo(Leu-Pro), $C_{11}H_{18}N_2O_2$ (This study)
F4 (7.8-8.1)[a]	211.0	55.2 (14.2), 58.2 (4.6), 68.2 (14.8), 69.2 (37.6), 70.3 (100), 71.3 (20.6), 83.3 (7.7), 86.3 (10.1), 97.3 (58.6), 98.3 (10.7), 111.3 (9.4), 112.3 (8.4), 114.3 (21.3), 125.4 (34.1), 126.3 (33.4), 140.4 (10.2), 154.4 (31.0), 168.5 (63.9), 194.5 (7.0), 210.6 (12.4)	cyclo(Leu-Pro), $C_{11}H_{18}N_2O_2$ (This study)

F5 (8.1-9.0)[a]	195.0	54.9 (14.2), 56.0 (13.5), 68.0 (14.1), 69.0 (25.2), 70.0 (100.0), 71.0 (10.3), 83.9 (17.6), 84.9 (13.6), 85.9 (10.0), 95.9 (10.3), 96.9 (21.7), 97.9 (8.2), 106.9 (28.7), 111.9 (23.9), 113.9 (39.3), 124.9 (21.3), 125.9 (9.9), 139.9 (7.6), 140.9 (4.2), 153.9 (10.5), 154.9 (2.0), 155.9 (4.6), 167.9 (45.3), 168.9 (8.5), 169.9 (10.2), 193.9 (41.0), 194.9 (5.9)	cyclo(Val-Pro), $C_{10}H_{16}N_2O_2$ (9) (This study)
F6 (9.5-10.5)[a]	261.0	261.2 (2.3), 260.2 (12.4), 244.2 (1.2), 224.2 (1.2), 155.1 (8.7), 154.1 (100.0), 153.1 (3.7), 147.1 (1.2), 125.1 (4.1), 114.1 (0.2), 108.1 (4.6), 107.1 (38.7), 91.1 (2.1), 77.1 (3.3), 71.1 (1.4), 70.1 (18.8)	cyclo(Tyr-Pro), $C_{14}H_{16}N_2O_3$ (Kwak *et al*., 2014)
F7 (12.2-13.2)[a]	197.0	196.2 (9.2), 181.1 (1.9), 169.1 (0.4), 168.1 (0.7), 167.1 (0.8), 156.1 (1.4), 155.1 (16.3), 154.0 (100.0), 153.1 (14.3), 152.1 (2.1), 140.1 (2.3), 139.1 (1.1), 138.1 (6.2), 137.1 (2.1), 125.1 (44.8), 114.1 (3.2), 113.1 (1.1), 112.1 (1.8), 111.1 (1.0), 110.1 (5.3), 107.1 (2.0), 100.1 (0.3), 99.1 (3.2), 98.1 (8.0), 97.1 (3.3), 96.1 (2.4), 84.1 (2.8), 83.1 (2.3), 82.1 (1.8), 76.1 (3.5), 73.1 (5.2), 72.1 (48.0), 71.1 (7.1), 70.1 (86.7), 69.1 (15.2), 68.1 (8.4)	*cis*-cyclo(L-Val-L-Pro), $C_{10}H_{16}N_2O_2$ (Kwak *et al*., 2014)
F8 (14.0-15.0)[a]	227.0	227.9 (5.1), 213.0 (1.26), 181.0 (7.60), 166.9 (3.2), 154.0 (11.8), 91.0(8.1), 77.0 (62.0), 61.0 (100.0)	*cis*-cyclo(L-Leu-L-Hyp), $C_{11}H_{18}N_2O_3$
F9 (15.0-16.5)[a]	185.0	154.0 (3.3), 149.0 (5.2), 141.0 (7.9), 129.0 (9.8), 128.0 (100.0), 127.0 (3.3), 113.0 (20.3), 112.0 (4.2), 99.0 (8.0), 98.0 (4.7), 92.0 (3.7), 91.0 (6.2), 86.0 (15.0), 85.0 (6.4), 84.0 (5.7), 83.0 (3.4), 71.1 (7.0), 70.1 (10.4), 69.0 (7.8), 60.0 (3.8), 57.0 (13.2), 56.0 (6.0), 55.0 (7.4)	cyclo(Ser-Pro), $C_8H_{12}N_2O_3$ (Kwak *et al*., 2014)

F10 (17.0-18.5)[a]	219.0	217.9 (12.6), 182.0 (3.0), 168.9 (5.4), 154.0 (77.1), 141.0 (8.1), 128.0 (41.2), 125.9 (24.5), 125.0 (13.9), 122.0 (11.4), 120.9 (12.6), 100.0 (17.2), 99.0 (13.5), 98.0 (12.0), 97.0 (8.2), 91.0 (34.4), 87.0 (37.1), 86.0 (17.8), 76.0 (100.0), 71.0 (11.2), 70.0 (47.3), 69.0 (25.3), 58.0 (11.3), 57.0 (42.6)	cyclo(Phe-Ala), $C_{12}H_{14}N_2O_2$ (This study)
F11 (19.0-20.0)[a]	261.0	260.1 (12.9), 244.1 (21.6), 169.1 (9.3), 168.1 (8.7), 167.1 (6.8), 155.1 (11.1), 154.1 (100.0), 153.1 (20.7), 152.1 (4.1), 141.1 (9.8), 125.1 (30.0), 120.1 (10.0), 114.0 (10.8), 111.1 (25.8), 107.1 (19.7), 98.1 (10.8), 97.1 (11.1), 92.1 (7.8), 91.1 (38.7), 86.1 (17.1), 85.1 (12.5), 84.0 (24.3), 83.1 (22.9), 82.1 (5.8), 81.1 (7.3), 77.0 (8.1), 74.0 (7.8), 73.0 (6.3), 72.1 (6.7), 71.1 (11.7), 70.1 (61.9), 69.1 (22.9), 68.0 (14.4), 67.1 (6.1), 65.0 (5.7), 60.0 (7.2), 57.1 (18.1), 56.0 (13.4), 55.0 (20.0)	cyclo(Tyr-Pro), $C_{14}H_{16}N_2O_3$ (This study)
F12 (20.5-22.0)[a]	211.0	210.0 (0.5), 195.0 (0.6), 181.0(1.2), 168.0 (0.6), 167.0 (2.0), 156.0 (0.8), 155.0 (8.8), 154 (100.0), 153.0 (5.3), 125.0 (11.9), 98.0 (2.8), 86.0 (18.2), 70.0 (48.5), 55.0 (3.3)	cyclo(Leu-Pro), $C_{11}H_{18}N_2O_2$ (Kwak *et al*., 2014)
F13 (22.5-23.5)[a]	211.0	166.1 (10.7), 154.1 (33.9), 148.0 (30.4), 135.1 (11.4), 125.1(3.0), 121.1 (12.2), 105.0 (19.0), 103.1 (13.2), 91.0 (100.0), 87.1 (66.0), 77.1 (13.5), 76.0 (40.2), 75.1 (22.5), 71.1 (7.7), 70.1 (20.9), 69.1 (76.2), 65.1 (12.1), 57.1 (20.7), 55.0 (7.0), 51.0 (6.4)	*cis*-cyclo(L-Leu-L-Pro), $C_{11}H_{18}N_2O_2$ (Kwak *et al*., 2013)
F14 (24.0-24.5)[a]	228.0	166.1 (10.7), 154.1 (33.9), 148.0 (30.4), 135.1 (11.4), 125.1(3.0), 121.1 (12.2), 105.0 (19.0), 103.1 (13.2), 91.0 (100.0), 87.1 (66.0), 77.1 (13.5), 76.0 (40.2), 75.1 (22.5), 71.1 (7.7), 70.1 (20.9), 69.1 (76.2), 65.1 (12.1), 57.1 (20.7), 55.0 (7.0), 51.0 (6.4)	cyclo(Met-Pro), $C_{10}H_{16}N_2O_2S_1$ (This study)

F15 (24.5-25.0)[a]	167	168.1 (0.3), 167.1 (1.9), 166.1 (13.6), 154.1 (5.4), 149.1 (7.5), 148.1 (58.6), 147.1 (10.4), 130.1 (2.6), 129.1 (1.9), 122.1 (2.1), 121.1 (15.9), 120.1 (4.2), 104.1 (2.9), 103.1 (22.9), 102.1 (1.7), 93.1 (2.3), 92.1 (27.2), 91.1 (100), 79.1 (3.6), 77.1 (13.6), 70.1 (4.2), 65.1 (21.9), 63.1 (4.9), 51.1 (9.3)	DL-3-phenyllactic acid, $C_9H_{10}O_3$ (This study)
F16 (26.0-27.0)[a]	245.0	244.0 (100.0), 154.0 (79.8), 153.0 (55.5), 125.0 (90.5), 91.0 (41.7), 70.1 (64.9)	cyclo(Phe-Pro), $C_{14}H_{16}N_2O_2$ (This study)
F17 (30.0-31.0)[a]	245.0	244.1 (97.5), 153.0 (82.0), 125.1 (100.0), 91.0 (52.3), 70.1 (46.3)	*cis*-cyclo(L-Phe-L-Pro), $C_{14}H_{16}N_2O_2$ (Kwak *et al*., 2013; Kwak *et al*., 2014)

[a] Retention time (min).

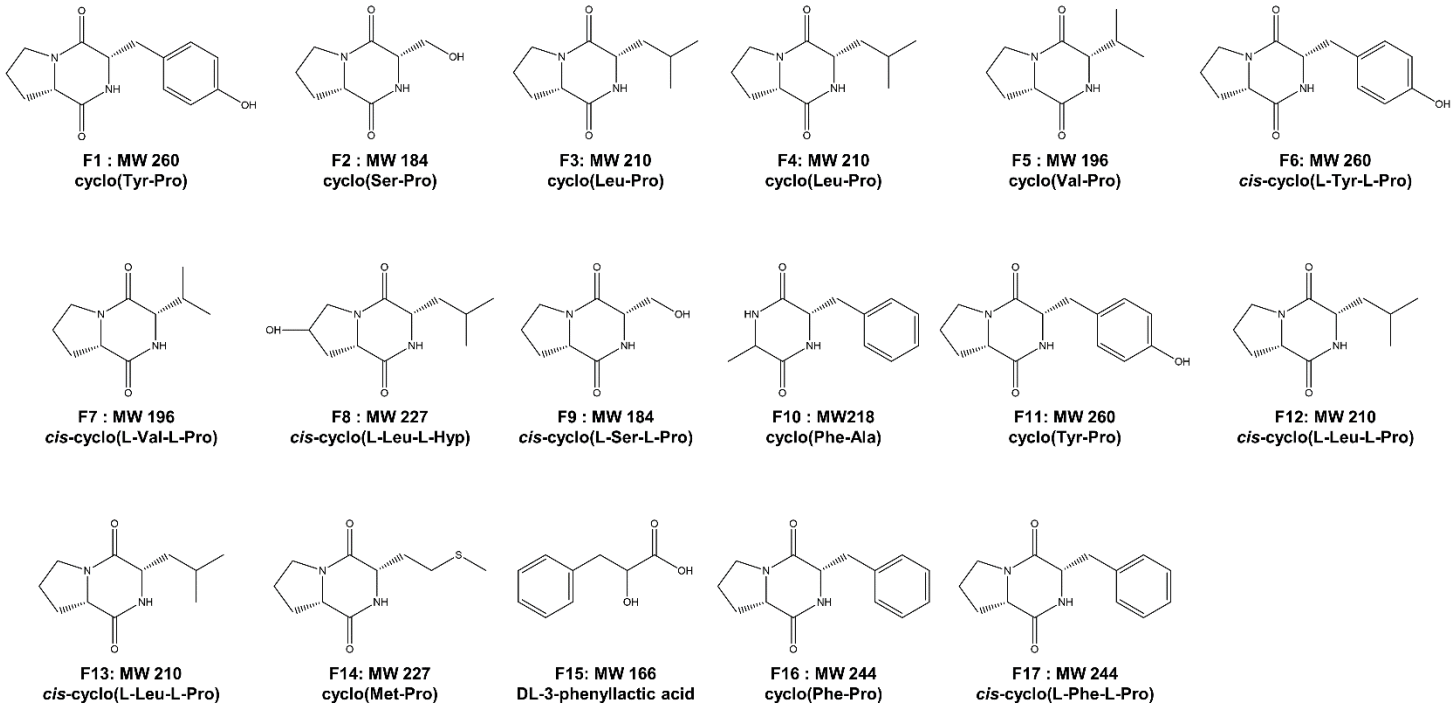


Fig. 5. Overall profiles of purified substances from *Lb. plantarum* LBP-K10 CFs. All fractions were identified as low molecular weight substances, including sixteen CDPs and one DL-3-phenyllactic acid by GC-MS experiments.

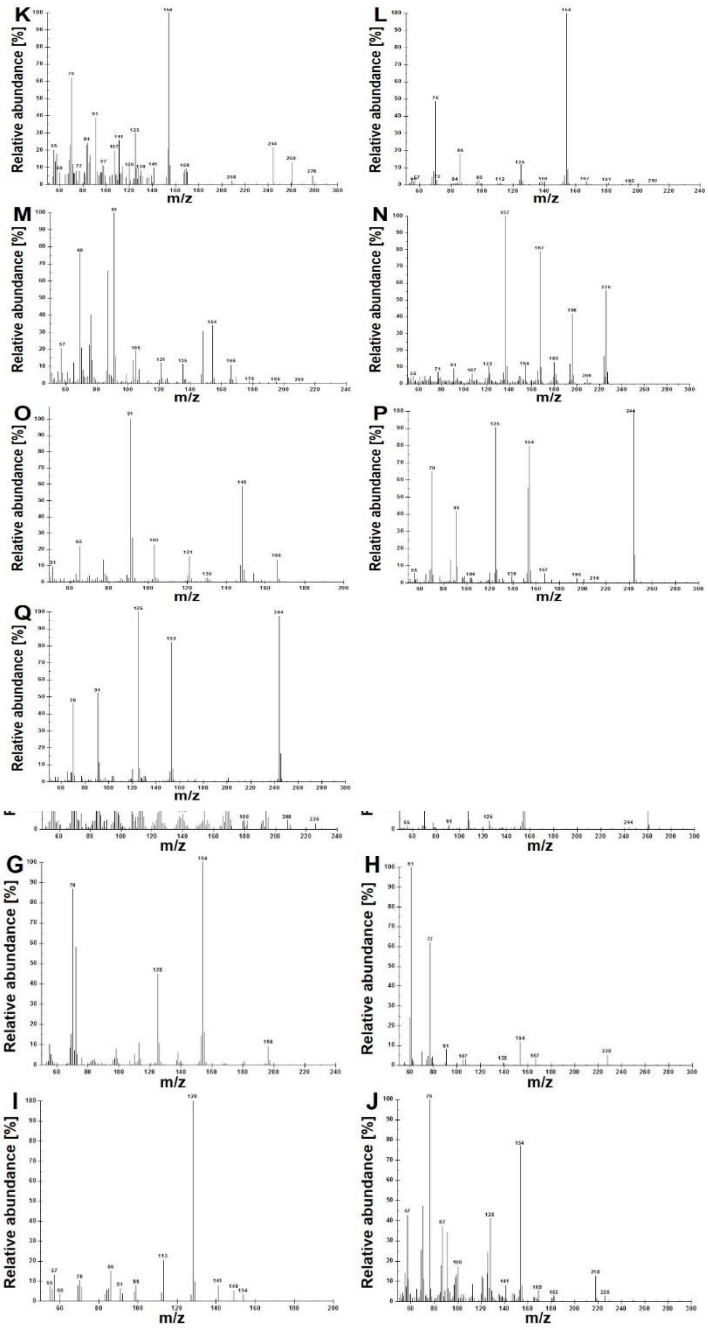

K
L
M
N
O
P
Q
F
G
H
I
J
Relative abundance [%]
m/z

Fig. 6. Structural analysis of seventeen fractions from *Lb. plantarum* LBP-K10. (A)-(Q) corresponding to F1-F17 obtained by GC-MS-EI/CI. The EI and CI values of each fraction were measured. From these fractions, the mass spectra of the following antibacterial, antifungal and antiviral substances are also shown: (A) F1, cyclo(Tyr-Pro); (B) F2 cyclo(Ser-Pro); (C) F3, cyclo(Leu-Pro); (D) F4, cyclo(Leu-Pro); (E) F5, cyclo(Val-Pro); (F) F6, cyclo(Tyr-Pro); (G) F7, *cis*-cyclo(L-Val-L-Pro); (H) F8, *cis*-cyclo(L-Leu-L-Hyp); (I) F9, cyclo(Ser-Pro); (J) F10, cyclo(Phe-Ala); (K) F11, cyclo(Tyr-Pro); (L) F12, cyclo(Leu-Pro); (M) F13, *cis*-cyclo(L-Leu-L-Pro); (N) F14, cyclo(Met-Pro); (O) F15, DL-3-phenyllactic acid; (P) F16, cyclo(Phe-Pro); and (Q) F17, *cis*-cyclo(L-Phe-L-Pro).

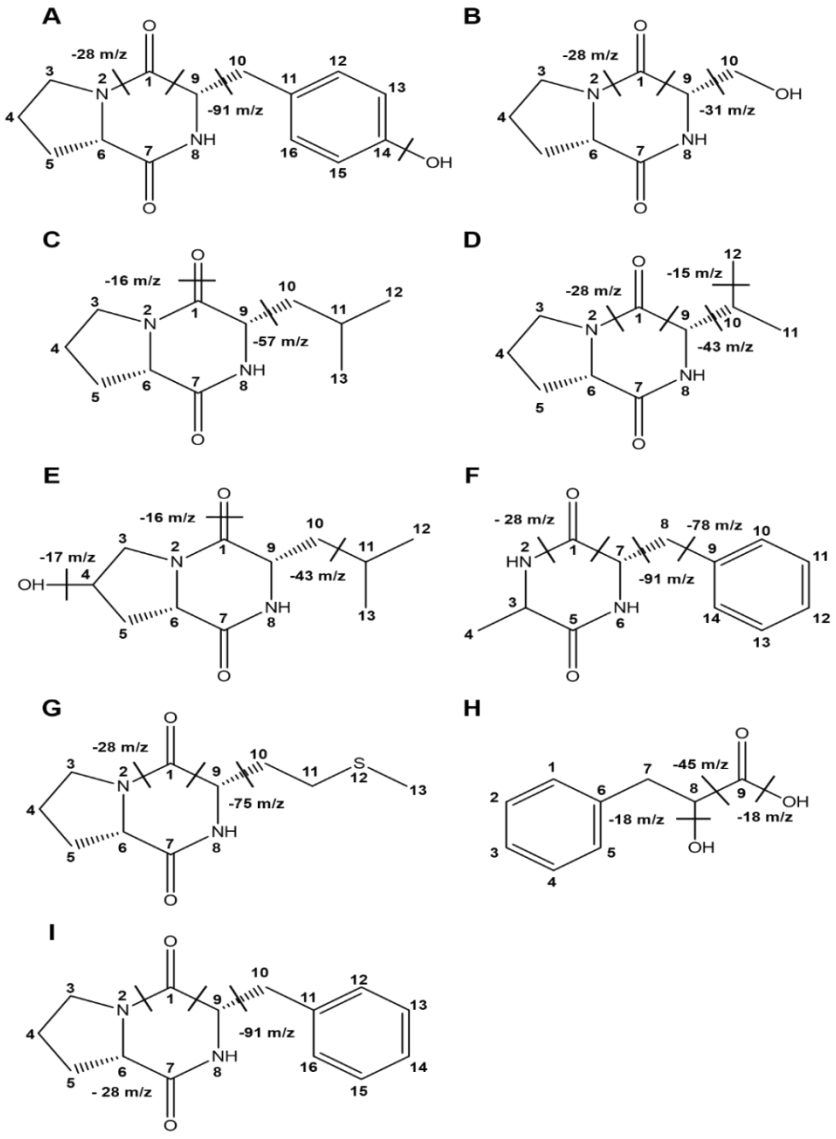


Fig. 7. The identified CDPs from F1 to F17 are summarised. The EI fragmentation patterns are shown as indicated. Structural units separated by chemical bonds are divided by dashed lines.

2. 3. 3. Antimicrobial activity of seventeen fractions

Due to the possible relevance to the conformational variation-related differences of bioactivity, we presumably hypothesized that the difference in the activity of different fractions that contain the same antimicrobial CDP might be caused by several types of diastereoselective or enantiomeric structures of CDPs as described. In the case of the synthesized CDPs, they could be interconverted by different diastereoselective or enantiomeric fashion influenced by pH, acylation and alkylation of chiral enolates (Borthwick 2012). It was also proven that a significant antibacterial activity of cyclo(D-Trp-D-Arg) contrast to a lower activity in the cyclo(L-Trp-L-Arg) might be due to the difference in the chirality properties of the tryptophan and arginine, which could form different spatial orientation that function as a crucial role in the enhancement of biological activities (Deepa *et al.* 2015). As predicted by stereochemistry that can play a pivotal role in the bioactivity of CDPs (Houston *et al.* 2004), a broad spectrum of antibacterial activity was commonly observed in the fractions containing proline based diketopiperazines, including F1, F3, F4, F6, F7, F8, F11, F12, F13 and F17 (Table 5). This antibacterial property driven by some types of CDPs corresponded to previous investigations particularly when using proline based cyclo(Leu-Pro) and cyclo(Phe-Pro) from *Lactobacillus* isolates, *Streptomyces* sp. KH-614 and *Ac. xylosoxidans*, respectively (Ström *et al.* 2002). Additionally, cyclo(Phe-Pro) and cyclo(Ile-Pro) from *Propionibacterium* CF showed antifungal activity against *As. fumigatus* and *R. mucilaginosa* (Lind *et al.* 2007). This observation also coincided with our previous investigations regarding the increased amount of poline-based fractions against microbes

(Kwak *et al.* 2013, Kwak *et al.* 2014), including cyclo(Tyr-Pro) (F6), *cis*-cyclo(L-Val-L-Pro) (F7), cyclo(Ser-Pro) (F9), cyclo(Leu-Pro) (F12), *cis*-cyclo(L-Leu-L-Pro) (F13) and *cis*-cyclo(L-Phe-L-Pro) (F17) (Fig. 3 and Fig. 2E). Therefore, all of the single CDPs potently inhibitory to bacteria, fungi and virus were summarized (Table 6), suggesting that the significant antibacterial activity against multidrug-resistant bacteria was shown particularly when treating *cis*-cyclo(L-Leu-L-Pro) (F13) (Tables 5 and 7). This result also corresponded to previous report regarding the effect of cyclo(L-leucyl-L-prolyl) and cyclo(L-phenylalanyl-L-prolyl) on the growth of five vancomycin-resistant enterococci (VRE) strains and pathogenic yeasts.

Table 5 Relative antibacterial activity of each fraction isolated from CF of *Lb. plantarum* LBP-K10

Strain	F1	F2	F3	F4	F5	F6	F7	F8	F9
[1] Antagonism test[a]*	+	–	++	+	+	+	++	+	+
[1] Antagonism test[b]*	+	–	++	+	+	+	++	+	+
[2] MIC[c]*	6.5	–	6.5	3.25	–	6.5	6.5	6.5	–
[2] MIC[d]*	6.5	–	6.5	3.25	–	6.5	3.25	3.25	6.5
	F10	F11	F12	F13	F14	F15	F16	F17	
[1] Antagonism test[a]*	–	+	++	+++	–	+	–	++	
[1] Antagonism test[b]*	–	+	++	+++	–	+	–	++	
[2] MIC[c]*	–	6.5	3.25	1.63	–	–	–	6.5	
[2] MIC[d]*	–	6.5	3.25	1.63	–	+	–	3.25	

[1] Symbol: +, <15 mm; ++, <22 mm; +++, >22 mm (Indicator strains: *B. subtilis* [a], *E. coli* [b])

[2] MIC: Minimum inhibitory concentration (Indicator strains: *B. subtilis*[c], *E. coli* [d])

* All experiments represent the average of three independent experiments.

Table 6 Antibacterial, antifungal and antiviral activity of the isolated CDPs from *Lb. plantarum* LBP-K10

Fraction	*m/z* of $[M+1]^+$	Molecule	Activity	Source or reference
F1	260.0	cyclo(Tyr-Pro)	antifungal	This study
F2	184.0	cyclo(Ser-Pro)	antifungal	This study
F3	210.0	cyclo(Leu-Pro)	antibacterial, antifungal and antiviral	This study
F4	210.0	cyclo(Leu-Pro)	antibacterial, antifungal and antiviral	This study
F5	194.0	cyclo(Val-Pro)	antifungal	This study
F6	260.0	*cis*-cyclo(L-Tyr-L-Pro)	antifungal	Kwak *et al*., 2014
F7	196.0	*cis*-cyclo(L-Val-L-Pro)	antifungal	Kwak *et al*., 2014
F8	226.0	*cis*-cyclo(L-Leu-L-Hyp)	Antibacterial and antifungal	This study
F9	184.0	*cis*-cyclo(L-Ser-L-Pro)	antifungal	Kwak *et al*., 2014
F10	218.0	cyclo(Phe-Ala)	–	This study
F11	260.0	cyclo(Tyr-Pro)	antifungal	This study
F12	210.0	*cis*-cyclo(L-Leu-L-Pro)	antifungal	Kwak *et al*., 2014
F13	210.0	*cis*-cyclo(L-Leu-L-Pro)	antibacterial, antifungal and antiviral	Kwak *et al*., 2013,Kwak *et al*., 2014
F14	227.0	cyclo(Met-Pro)	–	This study

F15	166.0	DL-3-phenyllactic acid	antifungal	Ström *et al*., 2002, This study
F16	244.0	cyclo(Phe-Pro)	antibacterial, antifungal and antiviral	This study
F17	244.0	*cis*-cyclo(L-Phe-L-Pro)	antibacterial, antifungal and antiviral	Kwak *et al*., 2013,Kwak *et al*., 2014

Table 7 Antibacterial activity of *cis*-cyclo(L-Leu-L-Pro) produced by *Lb. plantarum* LBP-K10

Indicator strains	MIC (mg/l) [a],*
Gram-positive bacteria	
Bacillus subtilis	12.42
Staphylococcus aureus	10.08
Listeria monocytogens	10.29
Streptococcus pneumoniae	9.81
Gram-negative bacteria	
Salmonella typhimurium	12.08
Escherichia coli	11.88
Shigella dysenterii	10.45
Multidrug-resistant strain	
Gram-positive bacteria	
Staphylococcus aureus 11471[b]	21.67
Streptococcus pneumoniae 14596 [b]	21.25
Gram-negative bacteria	
Salmonella typhimurium 12219 [c]	7.40

[a] MIC: Minimum inhibitory concentration.

[b] Multidrug-resistant Gram-positive bacteria.

[c] Multidrug-resistant Gram-negative bacteria.

* The values represent the average of three independent experiments.

2. 3. 4. K10-CCDP purification

Previously, we performed plaque assays to measure the antiviral activity of organic acid removed-CFs (Kwak *et al.* 2013), which had been successfully performed by modifications through Amberlite IRA-67 resin to isolate CDPs from CF for the first time. Particularly, we collected the unbound eluent from AEC using Purolite A420S to remove sugars with modifications, independently of using Amberlite IRA-67. Because the sequential use of the two resins was able to separate CDPs and remove both organic acids and sugars, the resulting total amounts of lyophilized compounds could be calculated through the AEC applications followed by MC extraction (Table 8). Interestingly, the resulting HPLC chromatograms of these three primary extracts showed a similar pattern compared to CFs from the isolated LAB (Fig. 8). After the fractions had been collected by HPLC, only fractions F1 to F17 that were sequentially re-extracted with MC and also the resulting three types of secondary MC extracts were lyophilized and re-quantified (Tables 9 and 10). Considering all of the data, we found that organic acids and sugars were effectively eliminated by three sets of AEC applications and the resultant lyophilized powders were used as bioactive K10-CCDP.

Table 8 Amounts of substances after the primary purification of K10-CCDP using AEC

Performance	Total quantity (mg/l)[a]
Culture filtrate without organic acids	
without methylene chloride extraction, a	28,097.5 ± 33.4
with methylene chloride extraction, b	497.5 ± 9.89
yield, % (b ÷ a ×100)	1.77
Culture filtrate without sugars	
without methylene chloride extraction, c	27,457.6 ± 22.4
with methylene chloride extraction, d	350.2 ± 5.92
yield, % (d ÷ c ×100)	1.28
Culture filtrate without organic acids and sugars	
without methylene chloride extraction, e	25,444.5 ± 28.8
with methylene chloride extraction, f	292.5 ± 4.68
yield, % (f ÷ e ×100)	1.15

[a] The values represent the average of three independent experiments.

Table 9 Total amounts of substances after the secondary purification of K10-CCDP by semi-prep HPLC followed by MC extraction

Identified compound	Total quantity in *Lb. plantarum* LBP-K10							
	Culture filtrate		K10-CCDP-I		K10-CCDP-II		K10-CCDP-III	
	mg/l[a]	μM	mg/l[a]	μM	mg/l[a]	μM	mg/l[a]	μM
F1	8.24±0.21	31.67±0.81	16.66±0.61	64.0±2.35	14.29±2.81	54.89±10.8	9.17±0.74	35.2±2.9
F2	2.88±0.04	15.64±0.22	1.78±0.13	9.66±0.68	1.59±0.13	8.65±0.69	1.5±0.06	8.15±0.31
F3	3.90±0.05	18.53±0.25	7.73±0.54	36.7±2.55	8.65±1.26	41.1±5.98	4.08±0.26	19.4±1.23
F4	1.05±0.20	5.01±0.96	1.62±0.01	7.69±0.47	1.38±0.26	6.57±1.24	1.26±0.05	5.95±0.25
F5	0.83±0.29	4.25±1.50	2.73±0.19	13.8±0.94	1.64±0.11	8.38±0.55	1.34±0.13	6.83±0.65
F6	16.1±0.08	62.0±0.30	14.0±2.34	53.8±8.98	12.9±1.21	49.67±4.66	10.43±1.72	40.0±6.62
F7	9.31±0.12	47.44±0.60	12.5±0.93	64.2±4.75	16.1±1.45	82.1±7.39	11.3±1.62	57.9±8.26
F8	3.04±0.05	13.41±0.21	6.62±1.74	29.3±7.69	9..39±1.94	41.5±8.58	5.6±0.27	24.74±1.18
F9	1.52±0.23	8.27±1.26	2.22±0.13	12.03±0.73	1.12±0.13	6.08±0.72	0.92±0.06	5.01±0.31
F10	0.98±0.13	4.49±0.59	10.63±1.37	48.7±6.27	15.4±0.89	70.8±4.06	8.3±0.74	38.0±3.39
F11	3.30±0.11	12.69±0.45	1.26±0.06	4.844±0.23	0.41±0.03	1.57±0.13	0.86±0.15	3.2±0.57
F12	4.32±0.14	20.5±0.67	2.69±0.03	12.7±0.12	2.67±0.01	12.7±0.05	2.38±0.17	11.3±0.82
F13	3.28±0.38	15.60±1.83	1.51±0.005	7.17±0.02	1.53±0.003	7.29±0.02	1.43±0.01	6.8±0.04
F14	2.09±0.05	9.15±0.24	0.92±0.07	4.01±0.3	1.14±0.03	5.03±0.14	0.85±0.39	3.71±1.72
F15	2.34±0.07	14.07±0.4	1.63±0.02	9.82±0.11	2.78±0.04	16.7±0.22	1.44±0.11	8.69±0.66
F16	1.68±0.08	6.87±0.32	1.74±0.23	7.11±0.97	0.19±0.01	0.77±0.05	0.12±0.02	0.48±0.07
F17	7.14±0.23	29.21±0.96	17.64±0.08	72.1±0.31	2.01±0.32	8.21±1.29	1.96±0.34	8.01±1.41

[a] The values represent the average of three independent experiment

Table 10 Summary of the total amounts of CDPs and DL-3-phenyllactic acid in K10-CCDP

Identified compound	Total quantity in *Lb. plantarum* LBP-K10							
	Culture filtrate		K10-CCDP-I		K10-CCDP-II		K10-CCDP-III	
	mg/l	μM	mg/l	μM	mg/l	μM	mg/l	μM
cyclo(Leu-Pro)	12.6	60.1	13.5	64.4	14.2	67.7	9.14	43.4
cyclo(Phe-Pro)	8.8	36.0	19.3	79.2	2.19	8.98	2.07	8.49
cyclo(Tyr-Pro)	27.7	106.3	31.9	122.6	27.6	106.1	20.4	78.6
cyclo(Ser-Pro)	4.4	23.9	3.99	21.6	2.71	14.73	2.42	13.1
cyclo(Val-Pro)	10.1	51.6	15.3	78.0	17.7	90.4	12.7	64.7
cis-cyclo(L-Leu-L-Hyp)	3.0	13.4	6.62	29.2	9.39	41.5	5.60	24.7
cyclo(Phe-Ala)	0.98	4.49	10.6	48.6	15.4	70.8	8.30	38.0
cyclo(Met-Pro)	2.09	9.15	0.92	4.01	1.15	5.03	0.85	3.71
DL-3-phenyllactic acid	2.34	14.0	1.63	9.82	2.78	16.7	1.44	8.69
Total	73.8		104.5		93.8		63.5	

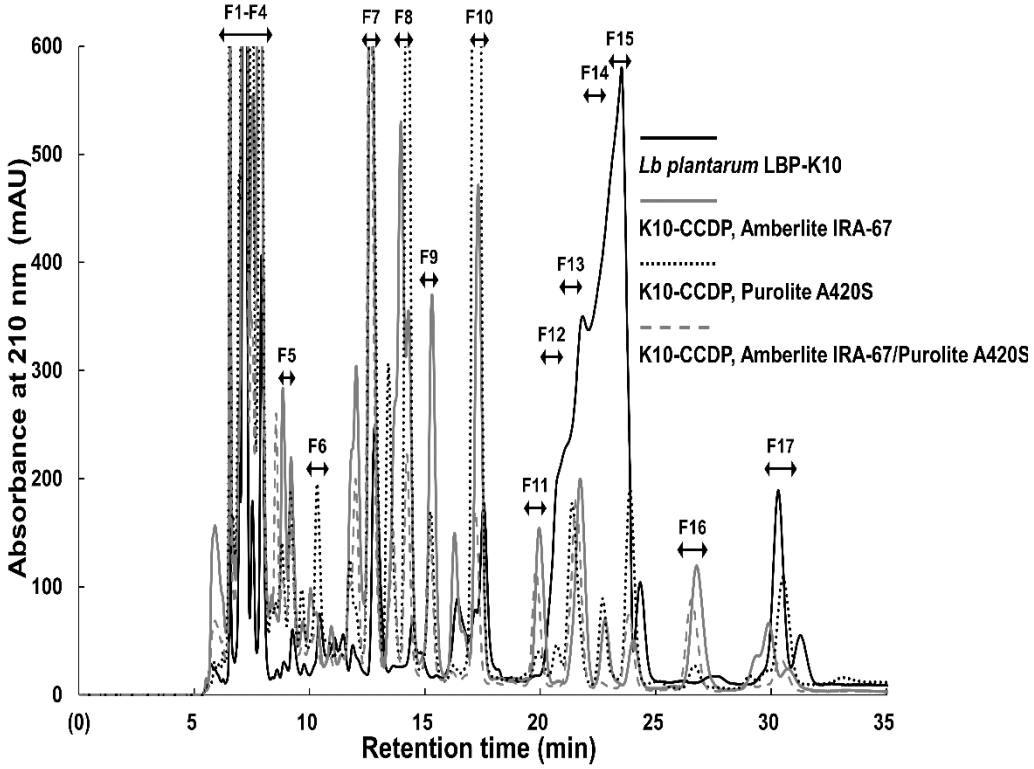


Fig. 8. Comparisons of the eluents from the CF obtained using Amberlite IRA-67 and CF of *Lb. plantarum* LBP-K10. Similar profiles of the CDPs of the eluents from the organic acid- or sugar-removed CF after the use of Amberlite IRA-67 or Purolite A420S and intact CF of *Lb. plantarum* LBP-K10.

2. 3. 5. Bioactivity of K10-CCDP against microbial and viral pathogens

K10-CCDP was observed to have significant antibacterial activity against reference strains and multidrug-resistant bacteria and its concentration that was required for bioactivity were shown by the types of the primary AECs (Table 11 and Table 12). In the case of the antibacterial activity of K10-CCDP-I against *S. aureus* 11471, *S. pneumoniae* 14596 and *S. typhimurium* 12219, the active *cis*-cyclo(L-Leu-L-Pro) (F13) levels in K10-CCDP-I were observed to be 0.27, 0.28 and 0.25 mg/l, respectively (Tables 9 and 112), which were revealed to be extremely lower than the active concentrations of 21.67, 21.25 and 7.40 mg/l for *cis*-cyclo(L-Leu-L-Pro) as a single compound (Table 7). These active *cis*-cyclo(L-Leu-L-Pro) (F13) concentrations in the K10-CCDP corresponded to decreases ranging from 30.77-, 25.94- and 41.25- fold to 90.10-, 75.96- and 120.79- fold for the dosages of K10-CCDP-I, K10-CCDP-II and K10-CCDP-III, respectively, compared to the use of the single *cis*-cyclo(L-Leu-L-Pro) (Tables 9, 10, 12 and Table 7). Cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) from *Streptomyces* sp. strain 22-4 were observed to be active against *Xanthomonas axonopodis* pv. Citri and *Ralstonia solanacearum* (Wattana-Amorn *et al.* 2015) with MIC of 31.25 mg/l, which corresponded to 1.09, 1.85 and 1.1 mg/l cyclo(Tyr-Pro) of three types of K10-CCDP (Table 10). Additionally, 12.5 mg/l *cis*-cyclo(L-Leu-L-Pro) from MIC values or the synergistic activity by the combination of CDPs using two types of proline-based CDPs against VRE strains, including *E. faecium* (vanA, vanB), and *E. faecalis* (vanA, vanB) with MIC values of 0.25–1 mg/l, and also against *E. coli*, *S. aureus* and *M. luteus*, were also significantly higher than that of single *cis*-cyclo(L-Leu-L-Pro) or *cis*-cyclo(L-Phe-

L-Pro) and K10-CCDP although the different pathogenic strains were used for antimicrobial activity (Rhee 2002).

The proliferation of *G. boninense* and *C. albicans* was also inhibited by treating three types of K10-CCDP (Table 12). The anti-*Ganoderma* or anti-*Candida* activity displayed 7.27-, 62.66 and 61.45-fold and 13.48-, 95.81- and 83.57-fold remarkably lower than 1.67 and 1.25 mg/l *cis*-cyclo(L-Phe-L-Pro) that were measured in our previous investigation (Kwak *et al.* 2014). Moreover, these active concentrations of K10-CCDP for fungal pathogens were also significantly lower than 20 mg/ml for cyclo(L-Ile-L-Pro) from the MIC values (Lind *et al.* 2007) and similar concentrations, as reported previously for cyclo(L-Phe-L-Pro), against *Fusarium sporotrichioides* or *A. fumigatus* (Ström *et al.* 2002). Morevoer, the antifungal activity of three types of K10-CCDP was more effective than the synergistic activity of lactic acid, cyclo(L-leu-L-pro) and cyclo(L-phe-L-pro) from *Lb. casei* AST18 (Li *et al.* 2012). Interestingly, even though it was observed that the combined effects of cyclo(L-leucyl-L-prolyl) and cyclo(L-phenylalanyl-L-prolyl) were especially effective against *C. albicans* and *C. neoformans* with MIC values of 0.25-0.5 mg/l , these active levels were also revealed to be extremely higher than those of K10-CCDP (Table 12).

Thus, the active concentration of each CDP in K10-CCDP was observed to be substantially lower than that in other previous studies that investigated the bioactivity of a single CDP or a certain of combination of CDPs against various bacterial or fungal pathogens. Furthermore, the active concentration of each CDP in K10-CCDP against influenza A virus (Table 13) suggests that K10-CCDP-III, which contained significantly

lower active concentrations of each *cis*-cyclo(L-Leu-L-Pro) or *cis*-cyclo(L-Phe-L-Pro), was observed to have more potent antiviral activity compared to other two types of K10-CCDP similar to antibacterial and antifungal activity.

Table 11 Antibacterial activity of K10-CCDP

Indicator strains	Active concentration of complex, MIC (mg/l)*		
	K10-CCDP-I	K10-CCDP-II	K10-CCDP-III
Gram-positive bacteria			
Bacillus subtilis	16.3	17.5	12.7
Staphylococcus aureus	16.3	17.5	12.7
Streptococcus pneumoniae	16.3	17.5	12.7
Gram-negative bacteria			
Escherichia coli	12.5	13.7	11.9
Salmonella typhimurium	12.5	13.7	11.9
Shigella dysenterii	12.5	13.7	11.9

* The values represent the average of three independent experiments.

Table 12 Comparison of the antimicrobial activity of K10-CCDP against multidrug-resistant bacteria and pathogenic fungi

Multidrug-resistant strains	Active concentration of complex, MIC (mg/l)[c]		
	K10-CCDP-I	K10-CCDP-II	K10-CCDP-III
Staphylococcus aureus 11471[a]	18.5	18.7	12.4
pneumoniae 14596 [a]	19.4	19.6	13.0
Salmonella Typhimurium 12219 [b]	17.2	17.4	11.5
Fungal strains	Active concentration of mg complex in 3 ml agar assay[c]		
	K10-CCDP-I	K10-CCDP-II	K10-CCDP-III
Plant pathogen			
Ganoderma boninense	37.0	34.0	24.8
Human pathogen			
Candida albicans	20.3	22.7	18.6

Multidrug-resistant [a] Gram-positive and [b] Gram-negative bacteria supplied by the Korea National Institute of Health.
[c] The values represent the average of three independent experiments.

Table 13 Antiviral activity of K10-CCDP against influenza A (H3N2) virus

Sample	Sample (%)(mg/l)	Plaque number[a]	(±SD)	Inhibition (%)
Untreated cells	0 (0)		76.6 ± 5.7 (8)	0
			82.1 ± 3.4	0
			79.4 ± 4.9	0
Cells treated with K10-CCDP	0.5 (7.176)	K10-CCDP-I	71 ± 2.6 (8)	7.31
		K10-CCDP-II	76.2 ± 2.6	7.19
		K10-CCDP-III	73.4 ± 6.5	10.08
	1.0 (14.352)	K10-CCDP-I	46.3 ± 4.6 (8)	39.56
		K10-CCDP-II	50.4 ± 4.1	38.61
		K10-CCDP-III	43.1 ± 5.5	45.72
	1.5 (21.528)	K10-CCDP-I	34.6 ± 6.1 (8)	54.83
		K10-CCDP-II	40.5 ± 4.7	50.67
		K10-CCDP-III	32.7 ± 4.2	58.82
	2.0 (28.704)	K10-CCDP-I	25 ± 2.6 (8)	67.36
		K10-CCDP-II	29.2 ± 2.9	64.43
		K10-CCDP-III	24.9 ± 4.6	71.17

[a] The values represent the average of three independent experiments.

2. 4. Discussion

It has been widely reported that bioactivity-based studies have been steadily updated to reflect the beneficial effects of CDPs from *Lactobacillus* spp. because of their rigid structure, quorum sensing and various physiological functions in organisms ranging from bacteria to humans (Wang *et al.* 2013). Hence, synthetic CDPs have been also suggested as strong candidates for drug design and human disease therapeutic applications (Borthwick 2012). However, although synthetic CDPs also seem likely to have potency, oral bioavailability and to be selective small molecules, the abiotic synthesis of CDPs has partially faced knowledge acquisition bottlenecks, such as complicated or expensive coupling reagents (Hulme and Gore 2003), difficult cyclisation (Martins and Carvalho 2007), synthesis that is mostly limited to small-scale solution (Dinsmore and Beshore 2002) and solid-phase reaction conditions (Fischer 2003). For this reason, high-throughput purification methods to obtain CDPs from CFs might be able to compensate for some of the difficulties in producing synthetic CDPs from the CFs. Moreover, a global CDP profile or a method for high-throughput quantitative purification of CF CDPs has not been obtained until now. Therefore, our current research aims to elucidate the CDP pool and its inhibitory effect against microbes and to develop an original method for the purification of the entire set of CDP analogues naturally present in the CF of LAB (Fig. 1).

It was suggested that the identical HPLC chromatographic pattern of the total pool of CDPs was commonly associated with excreting CDPs produced by LAB. The amount of all CDPs particularly increased to a greater extent in *Lb. plantarum* LBP-K10 cultures

than in those of other isolates. The present study also verified that the combination of CDPs exhibited a significant higher antibiotic activity against several types of pathogens compared with that of single CDPs. Although the bioactivity of single CDP was shown to be slightly similar to previous investigations, the active concentration of each compound in K10-CCDP was observed to be remarkably lower than that of the combined CDPs previously. These findings can provide a framework for future research or commercial fields such as agricultural pesticide, natural preservatives or animal feed additives and suggest the capability to assess the antibiotic effects of CDPs and their possible applications. In the case of the culture supernatant derived from this isolated strain, as shown in the CDPs content of the CFs (Tables 9 and 10), the LAB culture itself might be used in the feed stock containing various kinds of CDPs for domestic animals as eco-friendly feed stuff. Therefore, this study demonstrated that the single CDPs were bio-effectors but that the groups of CDPs could also exert potent bioactive effects against various types of pathogens. It has been also reported that the combination of CDPs could exert synergistic effects against microbes (Kwak *et al.* 2013). However, although the MIC and antagonistic effects were observed for each of the individual CDPs purified from *Lb. plantarum* LBP-K10 CFs in this study, we could not observe the combined mixtures of single CDP with each other due to the limitation of collecting each CDP by using a semi-preparative HPLC system. Hence, we had to consider the hypothesis on preparation of a massive amount of the complete set of CDPs from LAB CFs.

Herein, several types of fractions were observed to contain the same CDP, as indicated by the EI/CI data from GC-MS, despite these molecules being present in

separate peaks in the HPLC chromatograms (Fig. 6 and Table 4). This result suggests that the differences in retention times (Δt_R) of CDPs might be derived from the conformational enantiomeric pair or diastereomers, which were formed by an intramolecular hydrogen bond between the carboxy and carboxyamide in the possible cyclic dipeptidyl moiety (Brückner and Keller-Hoehl 1990). It was also demonstrated that the folded structures for instances of five diastereomeric pairs [c-(L-X-L-Y) and c-(L-X-D-Y)] were proposed to influence retention behaviour and lead to the separation of diastereomers on an reverse phase-HPLC column (Funasaki *et al.* 1993). In the case of cyclo(L-Ala-D-Ala), it was separated from the enantiomeric pair cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala) in 10 % methanol-contained mobile phase as proposed (Perzborn *et al.* 2013). Importantly, it was also suggested that the energy barrier to interconversion might be so high as to permit chromatographic separation of enantiomeric pair or diastereomers particularly of proline-containing peptides (Swadesh 2000). These previous reports presumably inferred that the isolated F1/F6/F11, F2/F9, F3/F4/F12/F13, F5/F7 and F16/F17 might be enantiomeric pair of the factions in this study (Table 4). Thus, our experimental results seemed to be the chromatographic fractionation of the racemic substances of the diastereomers, which could be entirely fractionated by MC extraction. However, other researchers have reported the antibiotic functions of bacteriocins and bacteriocin-like substances in the CFs from various types of LAB strains (Caplice and Fitzgerald 1999). Nevertheless, after the MC extraction of samples from repeated HPLC separation, all cases of isolated CDPs were sufficiently pure to be analyzed by GC-MS-EI/CI as one compound because any contaminant or

other substances were not observed under all experimental conditions (Fig. 4), indicating that the MC extraction of the CF was a purification method with strong selectivity for CDPs. Moreover, other experimental methods, such as 2D LC-MS/MS ESI at high- or low-resolution, did not show bacteriocin-like molecules (data not shown). Therefore, in this study, we focused on the purification of single CDP or sets of CDPs because we did not detect bacteriocin-like molecules or other compounds.

CHAPTER III.

CDPs prevent breast cancer

3. 1. Aims

- **The breast track cancer prevention effect of oral administration of CF-K10**
- **The breast cancer prevention effects of MC-K10**
- **The effect of MC-10 and cyclo(Phe-Pro) on breast cancer stem cells**

3. 2. Material and methods

3. 2. 1. Cancer cells

Human breast cancer cell MDA-MB-231 (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island. NY) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco), penicillin (100 U/ml, Gibco) and streptomycin (100 μg/ml, Gibco) at 37 ℃ in a CO_2 atmosphere.

3. 2. 2. Culturing and identifying lactic acid bacteria

CF-K10 were isolated, cultivated and identified as described in chapter II.

3. 2. 3. MC-K10 and cyclo(Phe-Pro) collection

The supernatant from three-day cultured *Lb. plantarum* LBP K-10 was collected by centrifugation, lyophilised, extracted with with 2-fold volumes methylene chloride (MC). After the methylene chloride fraction was evaporated, the residue were dissolved with distilled water, this mixture were named as MC-K10. To obtain cyco(Phe-Pro), MC-K10 were filtrated with a 0.22 μm-cellulose acetate membrane, and were separated using a semi-preparative HPLC system (Agilent, USA) with a semi-preparative Hypersil ODS C18 reverse-phase column (9.4 × 250 mm, Agilent, USA) and the ChemStation HPLC software.

3. 2. 4. Animal studies

All mouse studies were performed in accordance with protocols approved by the Animal Care and Use Committee at Southeast University (Approval ID: SEU-20150615-3). SCID mice were housed under pathogen-free conditions and were given autoclaved food and water. After determining the cell viability by trypan blue exclusion test, 2×10^6 MDA-MB-231 cells were injected into mammary fat pad in a 100 μl volume of sterile phosphate-buffered saline. SCID mice were used as 6 weeks of age and 10 mice for each group. CF-K10 were diluted 5 times with drinking water and given to the mice on the first day of cell transplantation. Tumors were measured using precision calipers twice weekly. Tumor volume was calculated at: volume =(length × width^2)/2.

3. 2. 5. Flow cytometry

After 3 washes with PBS, cells that were treated with MC-K10 or CDP and intact cells were detached with trypsin (Gibco) for 10 min at 37 ℃. For cell surface antigen phenotyping, floating and detached spindle-shaped cells were stained with fluorescent antibody CD133 (Becton–Dickinson, San Jose, CA, USA). Analyses were performed with FACS Calibur (Becton–Dickinson, NY, USA).

3. 2. 6. Cell-cycle analysis

2×10^6 cells were fixed in cold methanol, RNase-treated, and stained with propidium iodide (Sigma). Cells were analyzed for DNA content by EPICS-XL scan (Beckman Coulter) by using doublet discrimination gating. All analysis were performed in triplicate and 20,000 gated events/sample were counted.

3. 2. 7. Cell viability assay

Hoechst 33342 (Sigma-Aldrich, St. Louis) and Propidine Iodide (PI; Sigma-Aldrich) were used for staining cells treated in the mentioned condition following follow the method described before with slight modifications (Zhu *et al.* 2006). Cells were fixed for 30 min in phosphate-buffered saline (PBS) containing 1 % glutaraldehyde. After fixing at room temperature, the cells were washed twice with PBS and then exposed to 5 μg/ml Hoechst 33342 in PBS for 30 min at room temperature. The cells were then further incubated with 1 μl PI and were immediately observed using a fluorescence microscope.

3-4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) 50 μl of 2 mg/ml in 1 × PBS was added to each dish, and the indicated cells were incubated for 4 h at 37 ℃. The plates were then centrifuged at 500 × g for 10 min, and DMSO (120 μl, Sigma) was added to each well and incubated 1 h on an orbital shaker. The A570 nm was determined using an ultra-microplate reader (ELx 808; Bio-Tek Instruments, Winooski, VT).

3. 2. 8. Western blot analysis

Protein was extracted from the cells after culturing in the indicated conditions. After measuring the protein concentrations of homogenized lysates, 10 μg of protein extracted from cancer cells was separated by 10 % SDS–PAGE and transblotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking in a powdered nonfat milk

solution (5 % in PBS) with 0.05 % Tween-20, the blot was incubated with a polyclonal rabbit anti- human Bcl-2-associated death promoter (BAD) antibody (Cell Signaling, Danvers, MA), a rabbit anti- human caspase-3 antibody (Cell Signaling), a rabbit anti-human Bcl-2 antibody (Cell Signaling), a rabbit anti- human Cytochrome C antibody (Cell Signaling), a rabbit anti-human CD133 antibody (Cell Signaling), a rabbit anti-human Oct4/3 antibody (Cell Signaling) and a rabbit anti-human beta– actin antibody (Cell Signaling) at 1：1000 dilutions in 5 % blocking solution over night at 4 ℃. An anti-rabbit IgG antibody at a dilution of 1：5000 was used as a secondary antibody. The results were detected with an enhanced chemiluminescence kit (ECL; Amersham Bioscience/GE Healthcare, Little Chalfont, UK).

3. 2. 9. Sphere- forming assays

Cell monolayers were trypsinized to generate single-cell suspensions and counted by a hemocytometer. Single-cell suspensions (105 cells per 100 mm dish) were plated with an ultralow attachment surface. Three dishes were seeded for each cell line, and triplicate experiments were performed. Tumor spheres were counted and images of each well were taken on day 9. Immediately after the cells were seeded, each well was checked under the microscope to verify the sparseness of each spherical culture, and only wells containing single-cell suspension with no cell cluster were chosen for tumor-sphere counting after 9 days. Colonies of at least 60 μm in diameter (determined by using an eyepiece gratitude with crossed scales) were counted on day 9 after plating.

3. 2. 10. Statistical analysis

Data are presented as means ± standard error (SD) in quantitative experiments. The differences between groups were analyzed using the unpaired Student's test. P values <0.05 were considered significant.

3. 3. Results

3. 3. 1. Breast cancer prevention effect of CF-K10 in mouse model.

After centrifuging, the cell-free CF-K10 was diluted 5 times with drinking water. 1×10^6 MDA-MB-231 cells were injected in mammary fat pad of the mice. 20 % CF-K10 water was supplied on the first day of cancer cell transplantation. On the day of 31^{st}, the volume of the tumors in CF-K10 supplied group was significantly smaller than that in the positive control group. Actually, the obvious cancer inhibition effect could be observed on the 10^{th} day after cell transplantation, and this inhibition effect become significant on the day 17, meanwhile the tumor growth rate increased during the same time period. However, though CF-K10 water was cautiously supplied till the 31^{st} day, the tumor sizes were arrest since the 17^{th} day, while tumors keep growing in the positive control group (Fig. 9).

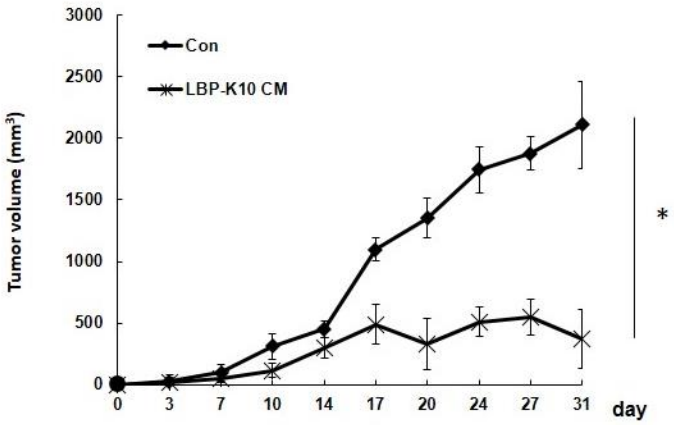


Fig. 9. CF-K10 inhibited the progress of in situ breast cancer progression in mouse model.

20 % CF-K10 diluted with drink water was supplied as daily drink water for the first day of transplantation of MDA-MB-231 cells in mammary fat pad of SCID mice. As early as on the 17th day, the volume of tumor was obviously different. On the 31th day, the volume of tumor showed a significantly difference between the 20 % CF-K10 supplied group and drink water supplied control group.

3. 3. 2. CF-K10 induced cell death on breast cancer cell.

The *in vivo* studies encouraged us to exploring the mechanism of breast cancer tumor prevention effect of the CF-K10. First, we treat the triple negative breast cancer cell line MDA-MB-231 with CF-K10. Majority of cells were lysed *in vitro* when cultured in conditions with more than 5 % CF-K10 supplement (Fig. 10). The viability of cancer cells were quantified with MTT assay, around 80 % of cancer cell could survive after 3 days with the treatment of 3 % CF-K10, and only 30 % of cells could survive in 5 % treatment (Fig. 11A). Further studies were executed in 3 % CF-K10. The cell cycle states were checked with FACs (Fig. 11B). The expressions of proteins that are evolved in apoptosis pathway, BAD, BCL-2 and cytosolic cytochrome C were examined with western blot. The results showed the expression of cytosolic cytochrome C and BAD were increased while BCL-2 expression level was decreased (Fig. 11C).The results revealed that cytosolic cytochrome C induce apoptosis may be active after 3 % CF-K10 treatment.

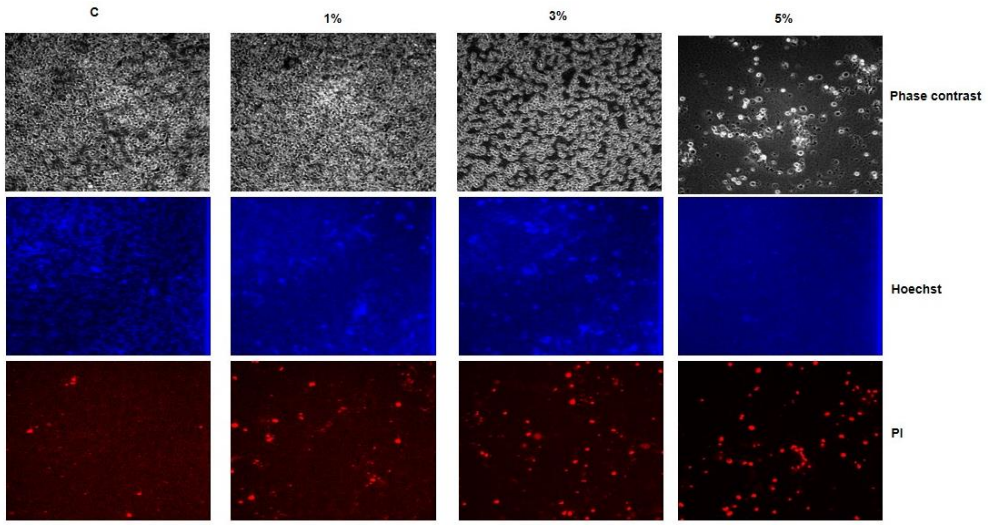


Fig. 10. CF-K10 induced apoptosis in breast cancer MDA-MB-231 cells.

Serial concentration of CF-K10 was employed to treat MDA-MB-231 breast cancer line. Hoechst 33342 (blue) and PI (red) staining were performed for identifying the death of cancer cells. Majority of cell dead with the treatment of 5 % CF-K10.

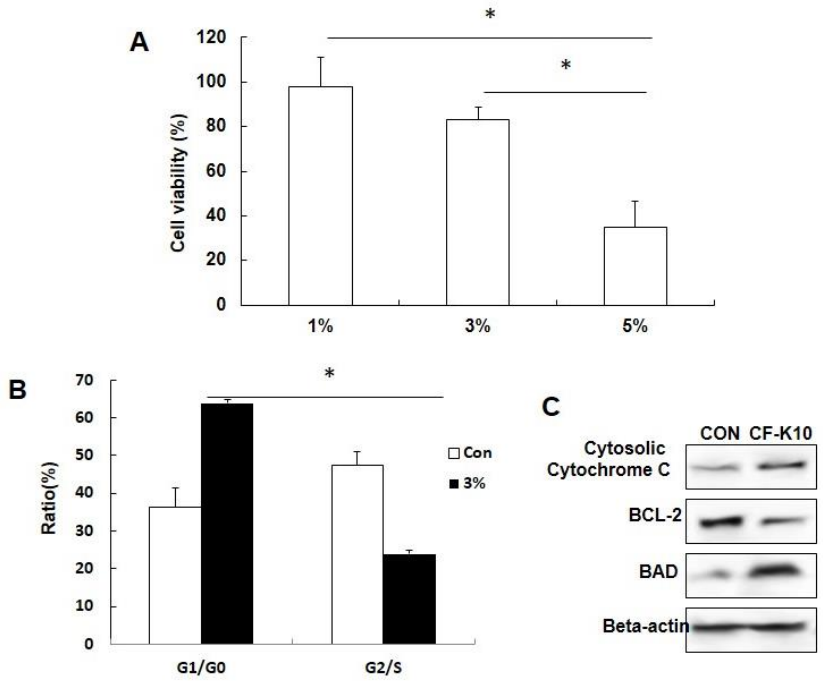


Fig. 11. CF-K10 induced cell growth arrest and activated apoptosis pathway.

MTT assay were performed to verify the cell viability in the present of different concentration of CF-K10. Only 5 % cancer cell survived in 5 % CF-K10 supplement condition (A). After three days of treatment of 3 % CF-K10, the 63.8 % of cells arrested in G2/S phase (B). The expression of apoptosis pathway related proteins were examined by western blot. The increasing expression of cytosolic cytochrome C and BAD and the decreasing expression of BCL-2 indicated CF-K10 induced the activation of apoptosis pathway in cancer cells (C).

3. 3. 3. CDPs complex effectively induced breast cancer cell apoptosis.

Our previous studies have displayed *Lb. plantarum* LBP-K10 isolated from fomented food produce several kind of CDPs. Single CDP components or MC-K10 has been proved to have antimicrobial effect even though the mechanisms was not well clearly elaborated yet. Since studies also proved that CDPs are capable of inducing cancer cell apoptosis, we wondered whether the MC-K10 derived from *Lb. plantarum* LBP-K10 possessed anticancer effect.

Separated MC-K10 was diluted to the original volume of CF-K10 with PBS. This solution was diluted with PBS as mentioned in further studies. Serial dilutions were tested on cancer cells. As shown in MTT assay, 57 % cancer cell can survive in 20 % MC-K10 supplement conditions, however, only around 10 % cancer cell can survive in 40 % MC-K10 supplement conditions (Fig. 12A). We further tested the expression of apoptosis related proteins, capase-3 and cytosolic cytochrome C. the expression of both proteins were elevated when the cells treated with 20 % MC-K10 (Fig. 12B). To further confirm caspase-3 pathway involved in MC-K10 induced apoptosis, Ac-DEVD-CHO, an inhibitor of caspase-3, were employed in MC-K10 treatment group. As a result, the survival rate was reversed to 82 % (Fig. 12C). These results indicated MC-K10 isolated from CF-K10 could induce breast cancer cell apoptosis through caspase-3 pathway.

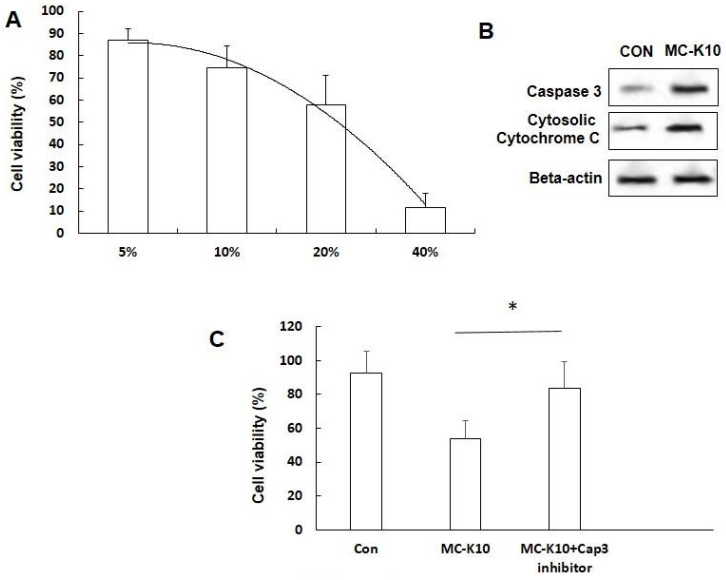


Fig. 12. MC-K10 induced caspase-3 dependent apoptosis in breast cancer cells.

Serial dilutions of MC-K10 with PBS were employed to treat cancer cells. MTT assay was performed to examine the viability of cells. The trend line also was also shown along with the column diagram. More than half of the cells can survive when the concentration of MC-K10 was less than 20 % (A).

Western blot showed caspase-3 elevated along with the increasing expression of cytosolic cytochrome C (B).

The cell viability reversed when caspase-3 inhibitor, Ac-DEVD-CHO was supplied in 20 % MC-K10 treatment group (C).

3. 3. 4. Cyclic dipeptides produced by *Lb. plantarum* LBP-K10 impaired cancer stem cells viability and tumor formation ability.

Cancers are initiated and maintain by cancer stem cells. Substance that can effectively shrink the tumor volumes may be resistance by cancer stem cells. Our *in vivo* and *in vitro* study encouraged us to test the effect of cyclic dipeptide CDP on cancer stem cells. As the most abundant component existing in CDPs, cyclo(Phe-Pro) was separated and purified this test. Serial sphere formation assay was performed. In the first passage of sphere formation test, the sphere formation ability was 43 % in cyclo(Phe-Pro) treated group when comparing with control group. However, the sphere formation ability in MC-K10 was significantly decrease to 45 %. There is a significant difference in sphere formation ability between passage-1 and passage-2 in cyclo(Phe-Pro) treated group, whereas , this sphere formation ability are comparable between passage-2 and passage-3 as around 40 %. The sphere formation of MC-K10 treated group was continuously decreased in passage-2 and passage-3 (Fig. 13A). Since CD133 was regarded as cancer stem cell marker, the partition of $CD133^+$ cells was analyzed. $CD133^+$ cells were decrease dramatically in MC-K10 treated group (Fig. 13B). We next examined another cancer stem cell marker, Oct4 and quantified the western blot results. Oct4 expression were decreased in both cyclo(Phe-Pro) and MC-K10 treated group. Compare with cyclo(Phe-Pro) treatment, the expression of Oct4 was dramatically down regulated (Fig. 13C and D). These results indicated MC-K10 could induce the stemness and self-renew ability of breast cancer stem cells.

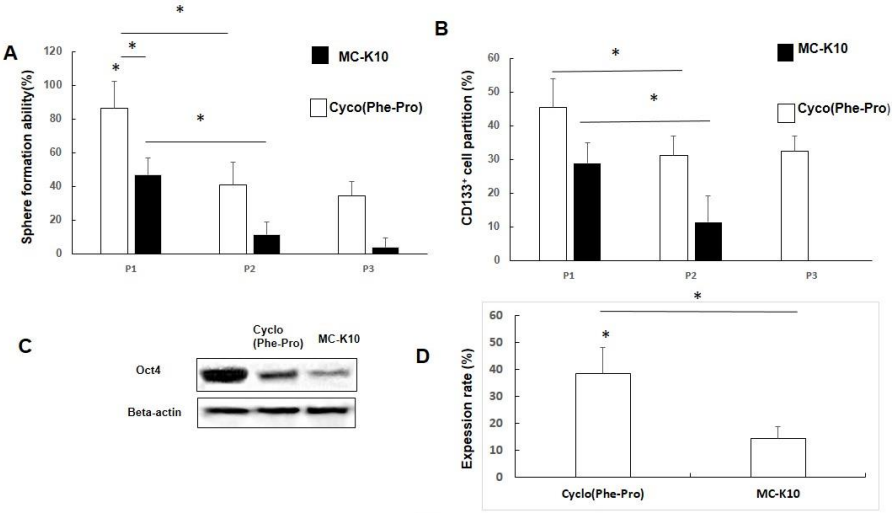


Fig. 13. Cyclic dipeptides impaired cancer stem cell activity.

10 mM cyclo(Phe-Pro) or 20 % CDPs was employed to treat MDA-MB-231 cells and the sphere formation ability was examined. Both the single cyclo(Phe-Pro) and MC-K10 were capable of impairing sphere formation, though MC-K10 showed a more strong inhibition activity(A). $CD133^+$ cell decreased dramatically in MC-K10 treated group, and due to few cells could obtain in passage-3, FACs was not possible performed in MC-K10 treated group. However, the $CD133^+$ cell partition in passage-2 and passage-3 was comparable in cyclo(Phe-Pro) treated group(B). Passage-2 sphere were lysed to examine the expression of Oct4 (C).

The western blot results of (C) were quantified. MC-K10 treatment significantly diminished the expression of Oct4 than cyclo(Phe-Pro) treatment, though cyclo(Phe-Pro) also can induce a dramatic decreasing expression (38.4 %) of Oct4 when comparing with intact control group (D).

3. 4. Discussion

LAB can survival in acid environmental and resist to the digest effect of bile, thus, it can pass through the GI track and adhere on the intestine cells. Since colon cancer is one of the major types of GI track cancer and LAB may directly interact with colon cancer in GI tack, studies focus on the anti-colon cancer effect of live LAB. Several mechanisms have been revealed. Very few studies investigated the effect of LAB or it product on non-GI track cancer. Previously, we confirmed a special set of cyclic dipeptide complex producing by *Lb. plantarum* LBP-K10, which give a strong effect of anti-microorganism. Despite the anti-microorganism effect, other studies confirmed cyclic dipeptides are capable of induce cancer cell apoptosis. Most importantly, studies revealed the quickly absorption of cyclic dipeptide in animal. Therefore, we purposed that oral up taking CF-K10 might inhibit the growth of non-GI track cancer, for example breast cancer. This study proved that oral in taking CF-K10 may effectively arrest the growth of breast cancer cell growth in animal model.

CF-K10 contained a large amount of organic acid that contribute to cancer prevention effect of *Lb. plantarum* LBP-K10, such as butyrate acid. Since the anticancer effect of organic acid has been well elaborated, we further tested the anticancer effect of MC-K10 secreted by *Lb. plantarum* LBP-K10. Consistent with the report and as our expectation, MC-K10 can effectively inhibit the growth of breast cancer cell.

Previously, we tested the effect of sodium butyrate, which is one of compound of organic acid complex produced by *Lb. plantarum* LBP-K10, on breast cancer prevention. The results indicated CMET expression in MDA-MB-231 may help the cancer cell to

resist sodium butyrate treatment. As a result, sodium butyrate treatment on MDA-MB-231 is difficult to eradicate breast cancer since the resistance effect of cancer stem cells on it. In this study, we interest on the effect of MC-K10 on cancer stem cells. Our results indicated MC-K10 treatment or the major compounds in this *Lb. plantarum* LBP-K10 conditioned CDPs, cyclo(Phe-Pro) may induce the apoptosis of breast cancer stem cells. However, the mechanisms need to be emphasized in detail, the *in vivo* result display that CF-K10 could not totally eradiated breast cancer, this may give a clue that another population of cancer stem cells may exist. Further studies need to be done for discovering the mechanism beneath these results.

Above of all, we proved that oral administration CF-K10 was helpful for inhibit the growth of in situ breast cancer in mouse model and CDPs participated in this inhibition effect. MC-K10 and cyclo(Phe-Pro) are capable of inducing apoptosis of cancer cells. Our study displayed the inhibition effect of CF-K10 on breast cancer by oral administration and CDPs administration produced by *Lb. plantarum* LBP-K10 was promising route for cancer therapy due to their inhibition effect targeting cancer cells.

CHAPTER IV.

A novel cyclic dipeptide synthase (CDPS) in *Lb. plantarum* LBP-K10

4. 1. Aims

- **CDPs synthesis through CDPS pathway**
- **Determination of CDPS amino acid sequence**
- **Overexpression of recombined CDPS and CDPs synthesis function examination *in vivo***
- **Over all CDPS Structure determination**

4. 2. Material and methods

4. 2. 1. CDPS purification from *Lb. plantarum* LBP-K10

The cultured cells were harvested by centrifugation at 12,000 rpm for 20 min and cell pellet was resuspended in 50 mM Tris-HCl buffer at pH 8.0 containing 1.0 mM phenylmethanesulfonylfluoride. The cell wall was disrupted by sonication using a W-225R Sonicator (Heat Systems-ultrasonics, Inc) with an appropriate amount of lysozyme (Sigma) at 30 sec each at a setting of 2.5 for 3 h on ice after repeating 10 times of the thawing and refreezing cell pellet. The resulting supernatant was collected by centrifugation and was used to purify CDPS. CDPS of cytosolic fraction was separated by DEAE-sepharose CL-6B by increasing the ionic strength of the eluting buffer ranging from 0 to 500 mM NaCl at 4 ℃. Each fraction was used to react with amino acids and ATP as substrates. MnSO4 and MgSO4 were also used to be minor compounds similar to the enzymatic conditions of Lactobacillus cultures. All solutions were prepared with the deionized and filtrated water (resistivity 18.2-MΩ cm at 298 K Milli-Q, Millipore).

4. 2. 2. CDPS activity assays

The CDPS activity was spectrophotometrically at 340 nm or fluorometrically measured by ninhydrin (2, 2-Dihydroxyindane-1, 3-dione) reaction with amino acids, which can cause to form chromophores, particularly using a method for activity staining after native PAGE with modifications (Shaykh *et al.* 1983, Friedman 2004). The 40 ml CDPS assay mixture for enzyme reactions consisted of 1.0 mg/ml CDPS and 1.0 mM

each of the amino acids, which were the combination of two amino acids link together to form CDPs, in 50 mM Tris-HCl, pH 8.0, respectively. CDPS activity was observed by the native PAGE using protein staining with ninhydrin. In both cell crude extract and the fractions of eluents driven by several types of column chromatographies. The native PAGE was performed and the resulting gels were briefly equilibrated with 50 mM Tris-HCl, pH 8.0 after rinsing in TDW twice for 10 min, respectively. Then, the gels were soaked with the reactant containing various types of amino acid pairs equilibrated with 50 mM Tris-HCl, pH 8.0. The resulting gels were stained with 25 mM ninhydrin and further incubated for 2 h. The archromatic bands were observed on native gels. Because the amino acids near CDPS were dehydrated, condensated and cyclized into CDPs, which lost the amino group, the achromatic bands could be shown as a blue or brown color. In contrast, other parts of the ninhydrin-based stained gels where did not show CDPS.

4. 2. 3. HPLC analysis

Active fractions containing CDPs resulting from enzyme reactions were concentrated by lyophilization and extracted with methylene chloride. After the elimination of methylene chloride by evaporation, the resultant was dissolved in distilled water and filtrated with a 0.22 μm-cellulose acetate membrane (Milli-Q, Millipore). The filtrated samples were separated by a high-performance liquid chromatography (semi-prep HPLC system, Agilent 1200 series) system with a semi-preparative C18 reverse-phase column (9.4 × 250 mm, Agilent, USA) and the ChemStation HPLC software using

UV absorbance with wavelengths at 210, 260 and 280 nm, respectively, as proposed previously (Kwak *et al.* 2013).

4. 2. 4. Overproduction of CDPS in *E. coli*

The gene sequence of CDPS with a calculated molecular weight of 26.1 kDa was amplified by polymerase chain reaction (PCR) using *Lb. plantarum* LBP K-10 genomic DNA as a template. The cloning of CDPS gene of *Lb. plantarum* LBP K-10 for PCR amplification was conducted by utilizing PCR primers as follows: 5'-CATATGGCAAAATTAGTATTGATTCGTCACGGT-3 (NdeI site, forward) and 5-GGATCCTTATTTGCCTAACTTTTCCTTACCAAG-3 (BamHI site, reverse) (NCBI accession number NP-391912.1). PCR amplification was performed with a Biometra thermocycler (Tampa, USA) for 30 cycles using amplification mixture contained 100 ng of genomic DNA, 0.5 μM of primer DNA, 0.2 mM dNTPs, 10 × Ex Taq buffer solution, and Taq polymerase (TaKaRa Bio Inc., Japan) 0.025 U/μl. PCR conditions were setted at 30 sec of denaturing at 95 ℃, 30 sec of annealing at 55 ℃, and 1.5 min of extension at 72 ℃. The PCR products was digested with NdeI and BamHI and inserted downstream of the pGEM T-easy vector (Promega). The resulting ligate was transformed to *E. coli* DH5α cells. The resulting pGemT Easy-CDPS construct was digested with NdeI and BamHI. NdeI- and BamHI-digested 690 bp CDPS gene was inserted into the pET3a (+) vector using TaKaRa's Mix ligation kit (TaKaRa Bio Inc.) the ratio of insert to vector was 5:1 (mole). After the ligation at 4 ℃ for overnight, the

E. coli BL21 (DE3) was used to transform the pET3a (+) vector containing *Lactobacillus* CDPS. The pET3a (+)-CDPS/BL21 (DE3) was incubated in LB (pH 7.4) with 50 μg/ml of ampicillin, while the control group BL21 (DE3) was incubated in LB (pH 7.4) (37 ℃, overnight) to obtain the seed cultures. 1 % (v/v) pET3a-CDPS/BL21 (DE3) culture was introduced to the LB (pH 7.4) added with 50 μg/ml of ampicillin and the control group BL21 (DE3) in LB (pH 7.4), and incubated (37 ℃, 220 rpm) for 2-2.5 h until the optical density reached a value of 0.4-0.5 at 600 nm. At the optical density points, isopropyl-beta-d-thiogalactopyranoside (IPTG, final concentration 1 mM, Sigma, USA) was added and further cultured to induce the CDPS protein synthesis (30 ℃, 150 rpm, 4-6 h).

4. 2. 5. Purification of CDPS in *E. coli*

The transformed pET3a-CDPS in BL21 (DE3) was cultured, harvested, centrifuged at 8,000 × g, 4 ℃ for 20 min. The cells were dissolved in lysis buffer, which consist of 50 mM Tris-HCl buffer, pH 8.0 at a ratio of 1:9 (w/v), 1 mM PMSF, and the cells was sonicated on ice for 5 min. After the cell debris was discarded by centrifugation at 12,000×g, 4 ℃ for 20 min), the filtrates was collected in a fresh tube and was used as an extract solution (Kanda *et al.* 1997). The quantity of protein was determined by the Bradford method (Bradford 1976) using the Bio-Rad protein assay reagent (Bio-Rad, USA). The resulting filtrates was loaded onto phenyl sepharose equilibrated with 1.0 x 10^3 mM ammonium sulfate. After the column was washed with the same buffer, the bound protein was eluted with a reverse linear gradient ranging from 1.0 x 10^3 to 0 mM

ammonium sulfate in 50 mM Tris-HCl buffer at a flow rate of 3ml/min. The resulting fractions were tested by native-PAGE using ninhydrin followed by combining and concentrating by ultrafiltration using PM10 membrane (Amicon). Subsequently, the concentrated enzyme solution was desalted using superdex75 FPLC system with 50 mM Tris-HCl (pH 8.0). The CDPS was further loaded on a DEAE-sepharose equilibrated with 50 mM Tris-HCl (pH 8.0) buffer. The enzyme was eluted with a linear gradient of 0-1.0 x 10^3 mM NaCl in the 50 mM Tris-HCl (pH 8.0) buffer. The purified enzyme was stored at 4 ℃.

4. 2. 6. 2D-LC-MS/MS analysis

The gel spots visualized by Coomassie Blue R-250 staining were excised, followed by destaining and reduction/alkylation with 10 mM and 50 mM iodoacetamide (Shevchenko *et al.* 1996). Following the dehydration of gel slices with acetonitrile, two volumes of freshly prepared trypsin (20 ng/mL^{-}1 in 25 mM NH_4HCO_3) were added and incubated at 37 ℃ for 18 h. Peptides were extracted with 10 ml of 0.1 % trifluoroacetic acid/ 50 % acetonitrile solution in two consecutive steps, dried through a vacuum centrifuge, and re-dissolved in 10 mL of 0.1 % trifluoroacetic acid. Mass analysis and protein identification were carried out on a ProteomeX LTQ 2D-LC-MS/MS spectrometer (Sinco, Inc.). The amino acid sequences were aligned using Clustal 2.1.

4. 2. 7. Molecular mass determination

The molecular mass of the purified CDPS was determined by gel filteraion

chromatography on a superpose 12 GL300 (HiLoad 16/60, 1.6 x 10 cm, GE Pharmacia), calibrated with alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), cytochrome c (12.4 kDa) aprotinin (6.5 kDa)

4. 2. 8. X-ray crystallography of CDPS

The CDPS was crystallized by the hanging-drop vapor diffusion method using 24-well costa plates at 295 K. The initial crystallization screening of CDPS was performed by the micro-batch method with Crystal ScreenI/II, Index, SaltRx. Natrix, Cryo, MembFac kits (Hampton Research, USA) and Wizard I, II screening solutions (Emerald Bio Systems). Droplets composed of 1 ul protein solution and equal volume of crystallization screening solution were loaded under layer of 1:1 mixture of silicon oil and paraffin oil in 72-well HLA plates (Nunc) and equilibrated at 295 K. Serval bundles of rod-shaped crystals were produced under the condition containing 100 mM HEPES pH 7.5, 50 mM magnesium chloride and 30 % polyethyleneglycol550 (PEG550) in three days. Then the crystallization condition was optimized with hanging-drop vapor diffusion method using 24-well well culture plate by adjusting from 30 % PEG550 to 16 % PEG550. Finally, single crystal was made in droplets containing 1 μl of protein sample (20 mg/ml) and an equal volume of precipitant solution containing 50 mM $MgCl_2$, 100 mM HEPES pH 7.5 and 17 % PEG550. The droplets were equilibrated against 400 μl of the same precipitant solution at 295 K and crystals grew to maximum size in 7 days.

The selenomethionyl CDPS was crystallized by the same procedures as the crystallization of native CDPS with a precipitant solution containing 50 mM $MgCl_2$, 100

mM HEPES pH 7.5 and 17 % PEG550. Native CDPS single crystals are soaked with 5 mM substrates (ATP, L-proline and L-phenylalaine) in crystallization condition to solve the cofactor binding complex structure.

4. 2. 9. Antimicrobial assays of CDPs produced by recombinant CDPS

Antimicrobial activity was investigated by using disk diffusion assay (Felten *et al.* 2002). Culture supernatant of recombinant *E. coli* were used and spotted on 6 mm paper disk (Toyo Roshi kaisha, 1td). Multidrug-resistant bacteria strains, used as indicator strains, was inoculated onto 1 % of the suitable molten agar. The spotted disk paper was putted on the agar plate and incubated 24 h at suitable temperature. Antimicrobial activity was estimated by inhibition zone diameter (mm).

4. 3. Results

4. 3. 1. CDPS activity and its verification

We examined the existence of CDPS by its synthesis ability in vivo. First, the crude extract from *Lb. plantarum* LBP-K10 was eluted with 300 mM NaCl through HPLC system and collected every hour, totally 11 fractions were obtained. Then, linear gradient of NaCl were performed for each fraction. After native SDS-PAGE loaded with each fraction was incubated with proline/Leucine or proline /phenylalanine with or without ATP, the gel was stained with ninhydrin. As our expectation, achromatic band could be observed on the blue stained gel in the ATP supplanted conditions (Fig. 14).We then tried to verify the generation of cyclic dipeptides. We applied all fraction of extract from *Lb. plantarum* LBP-K10 mix with amino acids and ATP. HPLC analysis confirmed the synthesis of *cis*-cyclo(L-Pro-L-Leu) and *cis*-cyclo(L-Pro-L-Phe). These results indicated the existence of CDPS in *Lb. plantarum* LBP-K10 conditioned medium as well as provided the clue that actively of CDPS was ATP dependent (Fig. 15C).

Fig. 14. The enzyme activity was verified by native activity staining.

Synthesis of cyclic dipeptide, catalyzed by CDPS, resulted an achromatic band appearing on the blue ninhydrin stained gel (indicated with red arrows)

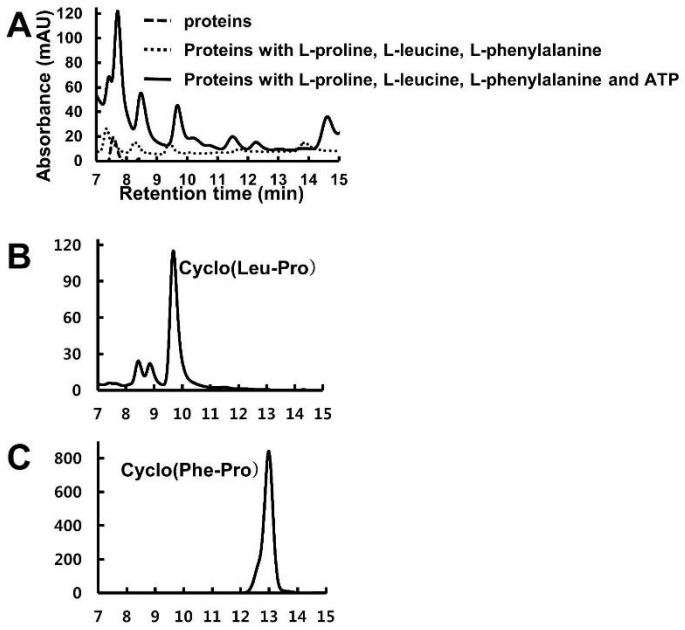


Fig. 15. HPLC analysis of cyclic dipeptides in reaction solution including enzyme and substrates. Enzyme catalyzed L-Proline/L-Leucine/L-Phenylalanine mixture to generating new substances, which were shown as peaks appeared after 9.7 min with present of ATP (A; Bond black line). However without ATP, Enzyme could not catalyze any reaction in the CDPS/L-Proline/ L-Leucine/L-Phenylalanine mixture (A; Dot line). Solutions contained the enzyme only were applied as control (A; Dash line). cis-cyclo(L-Pro-L-Leu) (B; MW 210) and cis-cyclo(L-Pro-L-Phe) (C; MW 244) were examined with HPLC, and the results indicated the existence of two cyclic dipeptide in the Enzyme/ L-Proline/ L-Leucine/ L-Phenylalanine mixture after reaction (Bond black line).

4. 3. 2. 2D-LC-MS/MS of *Lb. plantarum* LBP-K10 CDPS

According the results mentioned above, we confirmed the synthesis active native activity staining of fraction 5-11, which present a strong activity in figure14. The synthesis capability was increased and reach the strongest activity in fraction 9. Afterwards, the activity was gradually decrease (Fig. 14). Then, proteins fraction 5-11 were separated on SDS page for examine the candidate protein worked as CDPS. We proposed the synthesis activities should presented the concentration of CDPS proteins. Interestingly, the band indicated by red arrows present the same trends with the synthesis activity indicated as the band shown in A (Fig. 16).The red arrow indicated band (fraction 7-9) were cut and run on SDS-PAGE after stained with laemmli-staining method. Band of fraction 7 and 9 could be observed (Fig. 16B). Mass analysis and protein identification were carried out with the purified CDPS from fraction 9 band represented in Figure15A on ProteomeX LTQ spectrometer (Sinco, Inc). The purified CDPS like protein, could be identified as a polypeptide consisted of 230 amino acids with a calculated molecular mass 26.1 kDa (Table 1). A 690 bp of sequence from NCBI genome database could be obtained (Fig. 17 and Fig. 18). However, only an accession number as 11821150 was assigned. We then named this CDPS-like protein as cyclic dipeptide synthetase (CDPS) in this study.

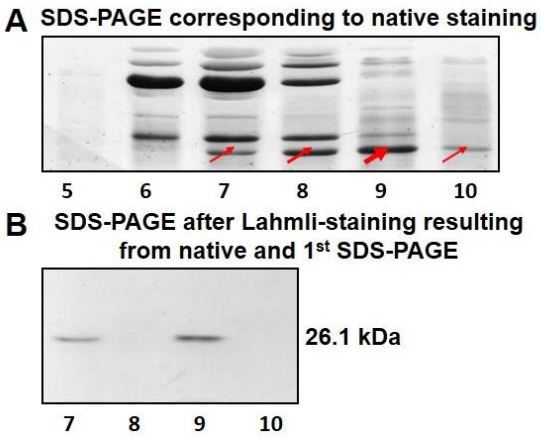
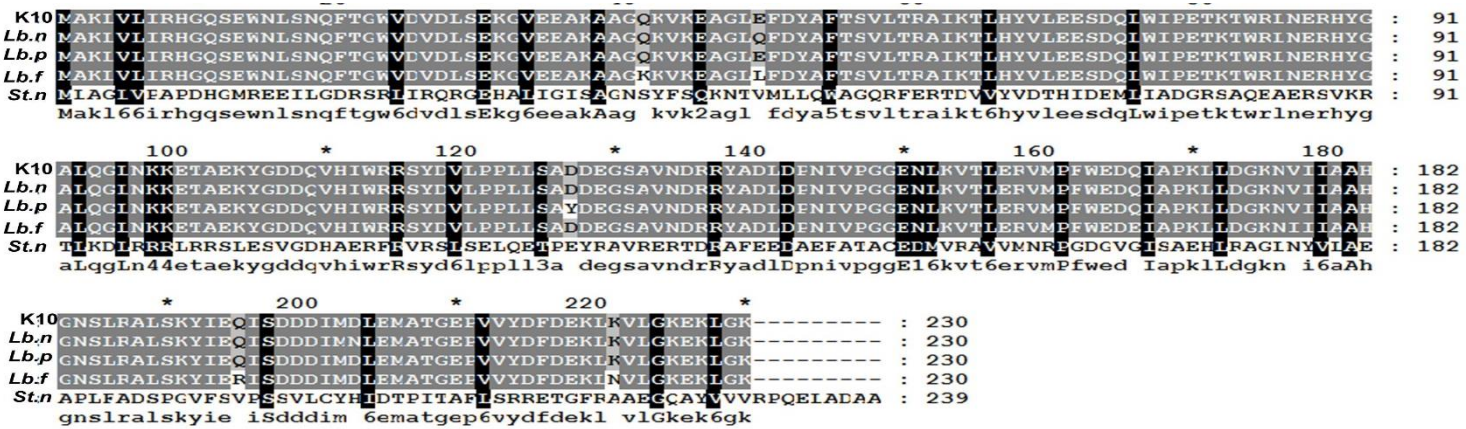


Fig. 16. Purification of enzyme in *Lb. plantarum* LBP-K10. (A) An achromatic band (red arrow) confirmed the protein existed in fraction 7-11 possessed CDPs synthesis ability (please refer to Fig. 14). The bands, whose level changing corresponding to native staining band in 14, were screen out and indicated with red arrows. (A). Laemmli-stained band of fraction 7-9 (indicated with red arrow in B) presented on SDS-PAGE again, the band from fraction 7 and 9 could be observed.

Fig. 17. The nucleotide and amino acid sequence of ORF of the cyclic dipeptide synthesis enzyme (NCBI gi: 311821850). A 690 bp sequence of the cyclic dipeptide synthesis enzyme was obtained from NCBI genome database



K10: *Lb. plantrum* LBP-K10; *Lb.n Lb. namurensis*; *Lb.p*: *Lb. plantarum*; *Lb.f*: *Lb. fabifermentans*; *St.n* : *Streptomyces noursei*

Fig. 18. Multiple sequence alignment of cyclic dipeptide synthetase in *Lb. plantarum* LBP-K10. The amino acid sequence deduced from the *Lb. plantarum* LBP-K10 cyclic dipeptide synthetase gene was aligned with other species using vector NTT9.0 explorer clustal X program.

Table 14 Results from 2D LC-MS analysis of purified CDPS

Accession	MW [kDa]	calc. pI	Score	Description
gi311821850	**26.1**	**5.07**	**1893.40**	**unnamed protein product [*Lactobacillus plantarum*]**
gi342240618	**30.9**	5.20	328.45	fructose-bisphosphate aldolase [*Lactobacillus plantarum* WCFS1]
gi334089837	36.4	5.54	188.77	glyceraldehyde-3-phosphate dehydrogenase [*Lactobacillus plantarum*]
gi300767613	25.7	4.64	138.68	triose-phosphate isomerase [*Lactobacillus plantarum subsp. plantarum* ATCC 14917]
gi342241915	47.1	4.88	84.67	30S ribosomal protein S1 [*Lactobacillus plantarum* WCFS1]
gi342242436	49.8	5.07	84.25	glucose-6-phosphate isomerase [*Lactobacillus plantarum* WCFS1]
gi342243047	47.2	5.64	78.17	adenylosuccinate synthase [*Lactobacillus plantarum* WCFS1]
gi342242987	39.9	5.78	67.88	inosine 5'-monophosphate (IMP) dehydrogenase [*Lactobacillus plantarum* WCFS1]
gi376010934	48.2	5.39	54.85	glutathione reductase [*Lactobacillus plantarum subsp. plantarum* NC8]
gi224831202	37.2	5.06	54.46	D-lactate dehydrogenase [*Lactobacillus plantarum*]
gi342240527	25.9	5.08	51.25	glucosamine-6-phosphate isomerase/deaminase [*Lactobacillus plantarum* WCFS1]
gi308180800	21.5	5.01	46.21	co-chaperone GrpE [*Lactobacillus plantarum subsp. plantarum* ST-III]

gi342241697	27.2	4.82	44.55	L-serine/threonine specific protein phosphatase [*Lactobacillus plantarum* WCFS1]
gi342241415	63.1	4.83	42.45	phosphoenolpyruvate-protein phosphotransferase [*Lactobacillus plantarum* WCFS1]
gi254555282	27.5	4.68	39.69	hydroxyethylthiazole kinase [*Lactobacillus plantarum* JDM1]
gi300636243	62.8	5.14	26.28	unnamed protein product [*Lactobacillus plantarum*]
gi46430487	33.9	4.98	25.74	L-lactate dehydrogenase [*Lactobacillus plantarum*]
gi45644480	57.4	4.81	22.95	GroEL [*Lactobacillus plantarum*]
gi308179245	23.8	5.02	22.56	beta-phosphoglucomutase [*Lactobacillus plantarum subsp. plantarum* ST-III]
gi254556766	96.5	5.34	20.49	ATP-dependent Clp protease, ATP-binding subunit ClpB [*Lactobacillus plantarum* JDM1]
gi2190422	40.7	5.59	19.72	alanine racemase [*Lactobacillus plantarum*]
gi376008932	25.9	5.21	19.58	D-ribitol-5-phosphate cytidylyltransferase [*Lactobacillus plantarum subsp. plantarum* NC8]
gi342242174	26.2	4.65	18.47	cell division initiation protein DivIVA [*Lactobacillus plantarum* WCFS1]
gi342241708	26.6	4.93	17.97	ribonuclease III [*Lactobacillus plantarum* WCFS1]
gi376009253	20.8	5.10	17.53	formylmethionine deformylase [*Lactobacillus plantarum subsp. plantarum* NC8]
gi254556111	53.2	5.33	16.91	aspartyl/glutamyl-tRNA amidotransferase subunit B [*Lactobacillus plantarum* JDM1]

4. 3. 3. Activity of recombinant CDPS

CDPS overexpression in pET3a (+) system in *E. coli* was firstly confirmed on SDS page stained with coomassie blue (Fig. 19). After the native gel loaded with CDPS was incubated for 4 h with several kinds of amino acids in the presence of ATP, the achromatic band was observed by ninhydrin staining (Fig. 19B).To confirm the overexpression of CDPS could result the generation of cyclic dipeptides, HPLC was applied for analyzing the total cyclic dipeptides produced by this gene transfected bacteria. According to the broad spectra of HPLC result, the expression of three L-Proline-based cyclic dipeptides, *cis*-cyclo(L-Val-L-Pro), cis-cyclo(L-Leu-L-Pro),cis-cyclo(L-Phe-L-Pro), was increased significantly (Fig. 20). After transfected with CDPS plasmid, the content of these fractions were remarkably increased up to 2.05-, 2.33- and 2.56-fold (Fig. 20 and 21). To confirm the *E. coli* transfected with pET3a-CDPS plasmid are capable of producing functional CDPS, disk diffusion assay was performed following the same method employed for *Lb. plantarum* LBP-K10 as mentioned above. As shown in Figure 22, CDPS transfected *E. coli* culture filtrates displayed antibacterial activity against gram positive and gram negative bacteria whereas control group did not. These data strongly suggested that CDPS transfection in *E. coli* results the functional target CDPS expression.

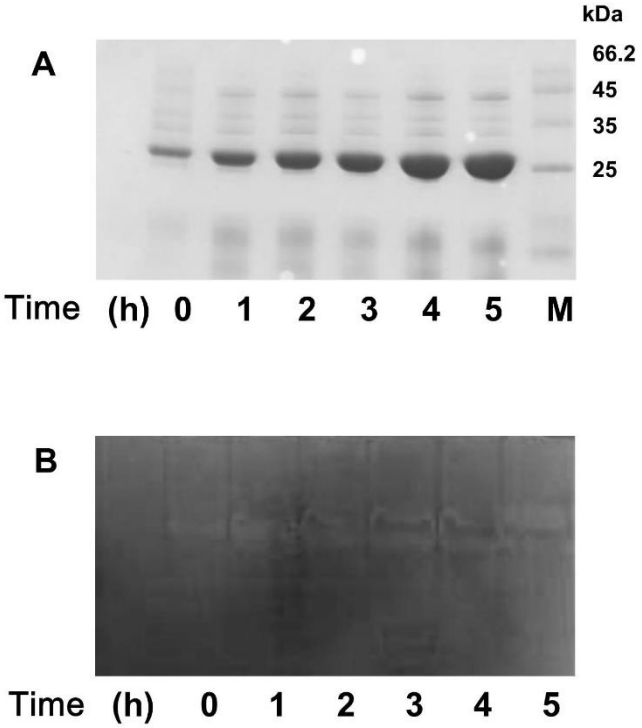
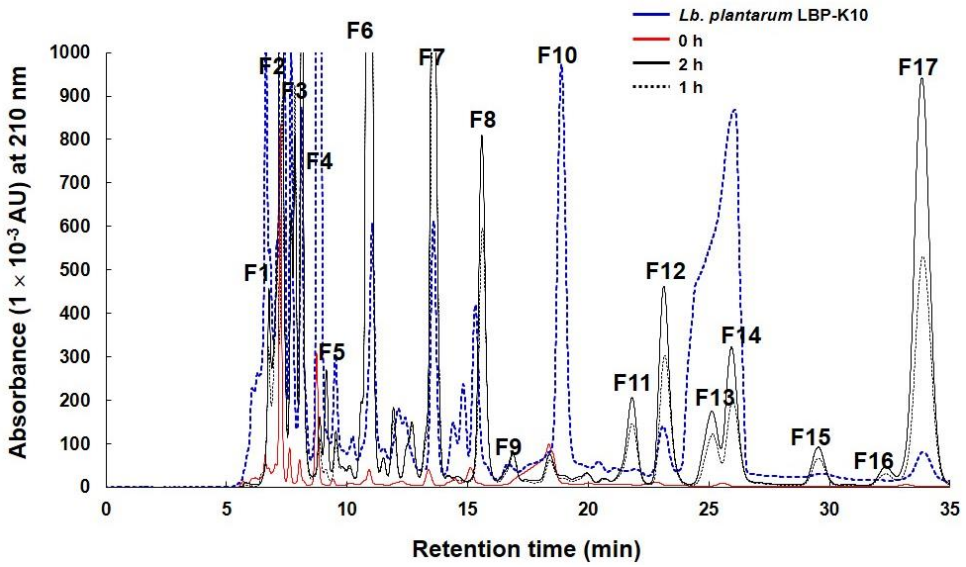


Fig. 19. Overproduction and native activity staining of CDPS. Overproduction of CDPS was verified by denaturing electrophoresis (A) and its function was confirmed with native activity staining (B).

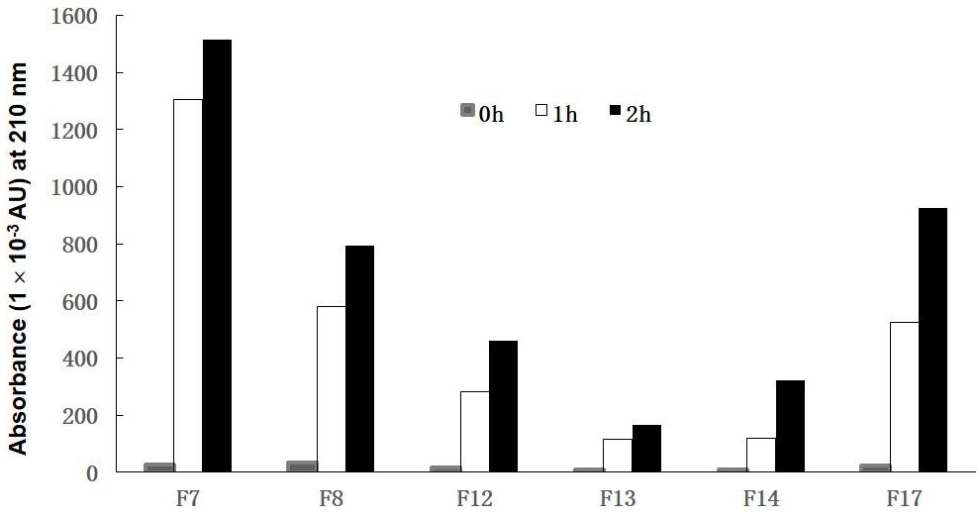


0 h: Incubated for o h after added with IPTG

1 h: Incubated for 1 h after added with IPTG

2 h: Incubated for 2 h after added with IPTG

Fig. 20. Cyclic dipeptides increased in culture filtrates of recombinant CDPS. To confirm the induction of cyclic dipeptides in transfected *E .coli* culture, HPLC analysis of culture extracts were performed. The result indicate, the most important functional CDPs of F7 (L-Val-L-Pro), F14 (L-Leu-L-Pro), F17 (L-Phe-L-Pro) increased significantly.

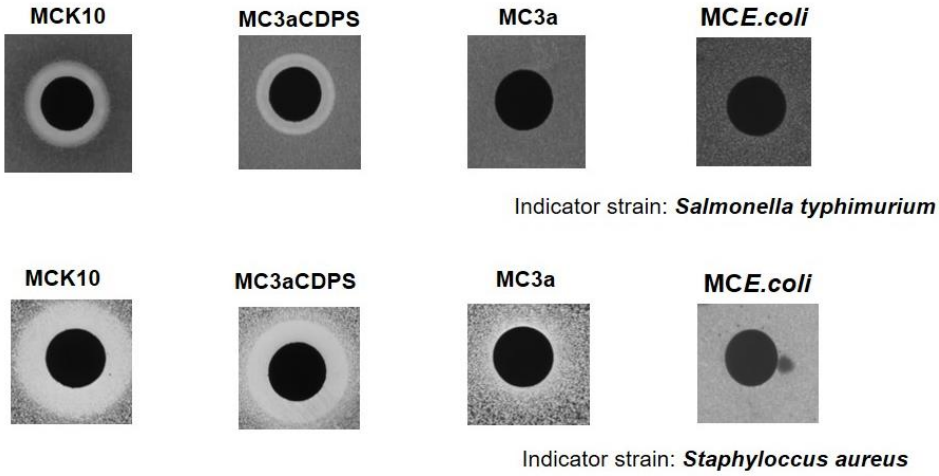


0 h: Incubated for o h after added with IPTG

1 h: Incubated for 1 h after added with IPTG

2 h: Incubated for 2 h after added with IPTG

Fig. 21. Overall profiles of the increased production of cyclic dipeptides using HPLC system in CDPS gene transfected bacteria.



MCK10: Culture supernatant of *Lb. plantarum* LBP-K10 were extracted by methylene chloride (MC).

MC3aCDPS: Culture supernatant of recombinant *E. coli* with CDPS were extracted by MC.

MC3a: Culture supernatant of recombinant *E. coli* without CDPS were extracted by MC.

MC*E.coli*: Culture supernatant of *E. coli* without CDPS were extracted by MC.

Fig. 22. MC extract of transfected *E. coli* medium showed enhanced antibacterial activity. After transfected with CDPS plasmid, the antimicrobial activity of MC extract from *E. coli* was increased and was comparable with MCK10. However, the extract from control plasmid transfected or intact *E. coli* display a weak or negligible effect on the bacteria indicators.

4. 3. 4. Detection of cyclic dipeptides produced by purified enzyme

To confirm the recombined over expressed CDPS was functional, cyclic dipeptide synthesis activity was tested *in vitro* or *in vivo*. CDPS were purified with superdex 75 chromatogram and collected every hour. Each fraction run on SDS-page the over expressed 25 kDa band were confirmed. Consistent with our previous results, F9 fraction contains the most enriched CDPs (Fig. 23A). Synthesis function analysis followed by ninhydrin staining of purified CDPS were performed as described as before. Through the enzyme reaction and consecutive activity staining, the active bands where CDPS located showed transparent grey color while the background generally present blue color (Fig. 23B). The presence of cyclic dipeptides synthesized by purified enzyme *in vitro* was further confirmed by HPLC. In HPLC analysis conditions, the impurity or solvent peaks were detected within ten min. Moreover, the retention time of cyclic dipeptides stands were always shown up longer than 10 min. Therefore, the peaks which were detected by HPLC chromatogram with a retention time more than 10 min were regarded as cyclic dipeptides or other byproducts through the enzyme reactions. These results indicate that the predicted cyclic dipeptide fractionation driven by the enzyme reactions could be performed. Because the optimal temperature for bacteria biological activities is 37 ℃ and the activity of CDPS was ATP-dependent, pH valve of the reaction solution might be the most important factor to be concerned in the reaction conditions. Therefore, several pH conditions were intensively examined using each amino acid combinations including L-Proline and L-Lysine, L-Proline and L-Phenylalanine, L-Proline and L-Serine. The amount of the cyclic dipeptides

produced by enzyme reactions in the *in vitro* condition was measured by HPLC chromatograms. Interestingly, the most efficient pH values for enzyme activity were delicately different by the amino acids combination-dependent manner. Therefore, CDPS activity was significantly higher in pH 8 than in other pH. pH 8 was the most optimal reaction pH condition for the production of cyclo(Pro-Lys) and cyclo(Pro-Val) with the highest peak of cyclic dipeptide presented on the HPLC spectra (Fig. 24-1, Fig. 24-4) contrast to the low activity at pH 6 for producing cyclo(Pro-Ser) (Fig. 24-3). When comparing to reference experiments, HPLC chromatograms explained that CDPS could contribute to the generation each cyclic dipeptide such as Pro-Lys, Pro-Phe, Pro-Val, Pro-Ser and Pro-Leu) (Fig. 24).

To confirm the peaks in HPLC represented are cyclic dipeptides, we collected each compound according each peak, and then subjected them for GC/MS analysis using electron ionization and chemical ionization. With the reference we have obtained from the data base and the compound isolated from lactic acid before, we concluded that cyclo(Phe-Phe) and cyclo(Pro-Phe) could be generated in the reaction system including L-Proline and L-Phenylalanine (Table 15-1), cyclo(Pro-Lys) and cyclo(Lys-Lys) could be found in the reaction system including L-Proline and L-Lysine (Table 15-2), cyclo(Pro-Ser) in the system including L-Proline and L-Serine (Table 15-3), cyclo(Pro-Val) in the system including L-Proline and L-Valine. However, we could not exclude the possibility of the existing of other kind of cyclic dipeptides, which could be undetectable due to small amount or inefficiency ionization.

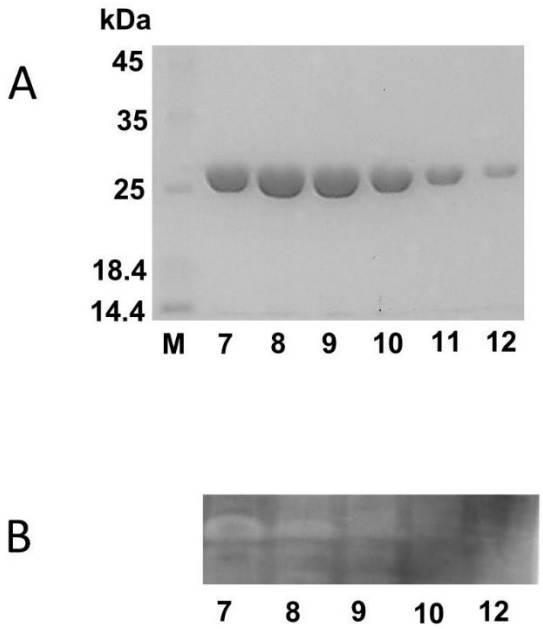


Fig. 23. Purification and activity assay of Recombinant CDPS. After gel filtration with superdex75 column chromatography, recombined CDPS were confirmed on SDS-PAGE (A). Activity of purified CDPS were checked using native staining assay (B).

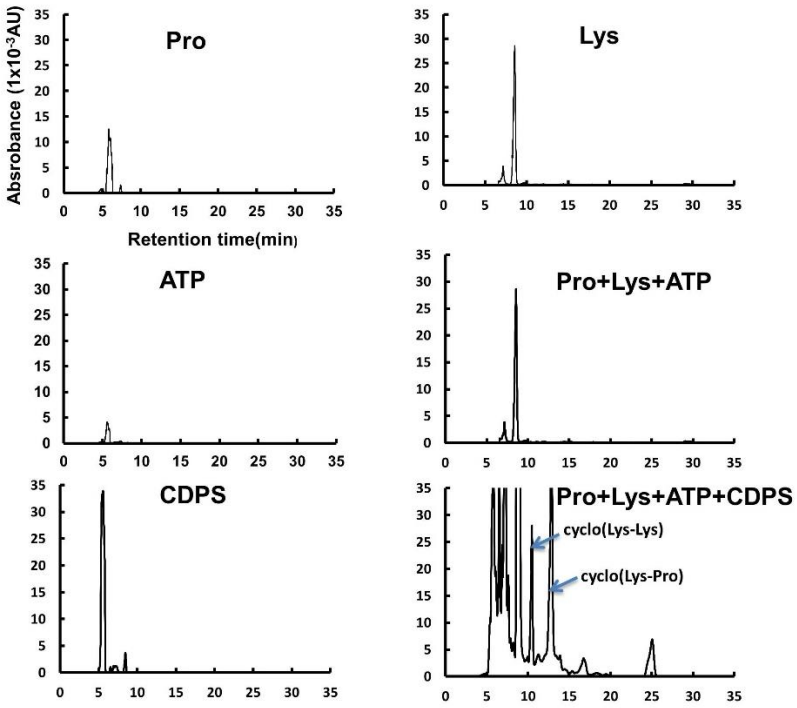


Fig. 24-1. HPLC analysis confirmed CDPS that catalyzed the synthesis of cyclo(Pro-Lys) *in vitro*. CDPS catalyzes two amino acid pairs, such as L-Proline and L-Lysine, to generate cyclo(Pro-Lys) *in vitro*. The chromatograms peaks of L-Proline (upper left), L-Lysine (left right), ATP (middle left) and CDPS (lower left) were shown as indicated. In the case of the reaction mixture of L-Proline, L-Lysine and ATP, it did not show CDPs peaks within 10 min (middle right). However, when CDPS was added to the reaction mixture, the biosynthesized cyclo(Pro-Lys) and cyclo(Lys-Lys) (as indicated in the figure) was observed as new peaks ranging from 16.5-16.7 min.

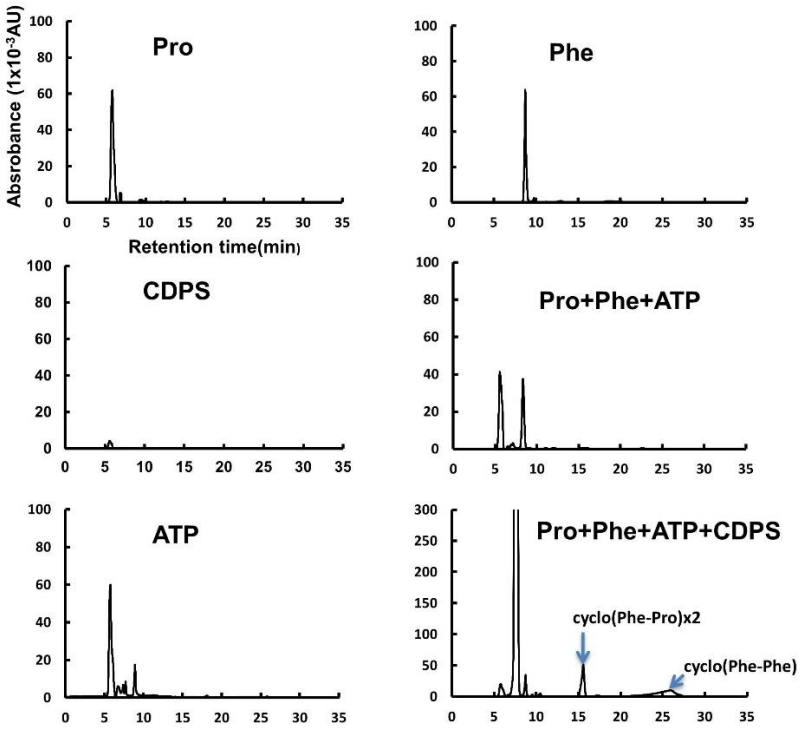


Fig. 24-2. HPLC analysis confirmed CDPS that catalyzed the synthesis of cyclo(Pro-Phe) *in vitro*. CDPS catalyzes two amino acid pairs, such as L-Proline and L-Lysine, to generate cyclo(Pro-Lys) *in vitro*. L-Proline (upper left), L-Lysine (left right), ATP (middle left) and CDPS (lower left) peaks were shown as indicated. In the case of the reaction mixture of L-Proline, L-Lysine and ATP, it did not show CDPs peaks within 10 min (middle right). However, when CDPS was added to the reaction mixture, the biosynthesized cyclo(Pro-Phe) and cyclo(Phe-Phe) was observed as new peaks ranging from 15.5-26 min.

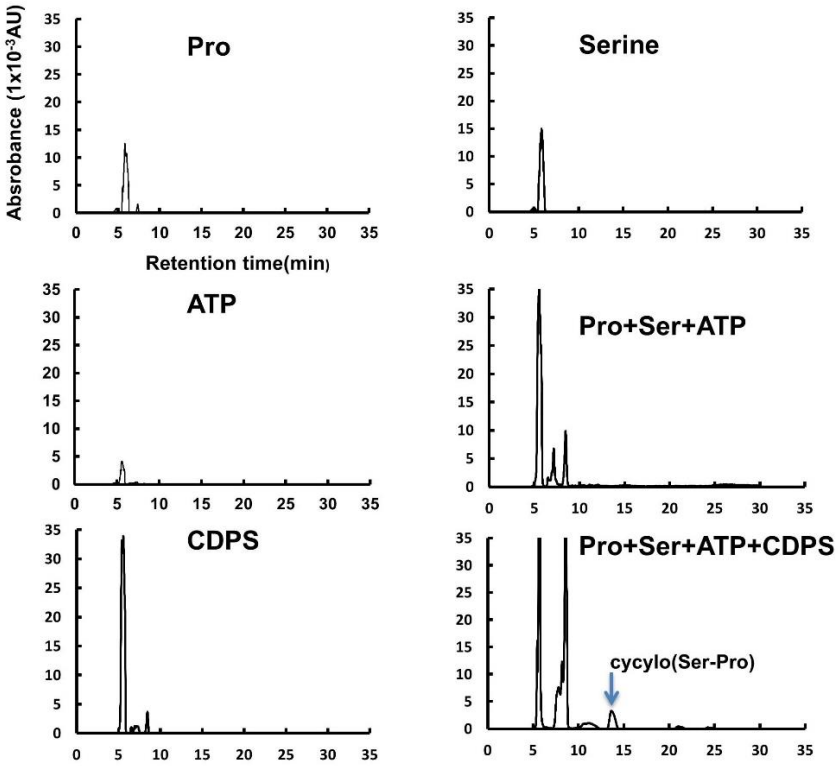


Fig. 24-3. HPLC analysis confirmed CDPS that catalyzed the synthesis of cyclo(Pro-Ser) *in vitro*. L-Proline (upper left), L-Serine (left right), ATP (middle left) CDPS (lower left) peaks were shown in HPLC chromatogram. The reaction mixture of L-Proline, L-Serine and ATP presented no peaks after ten min (middle right). However, when CDPS was added, a peak of new substance [cyclo(Pro-Ser)] appeared around 15 min.

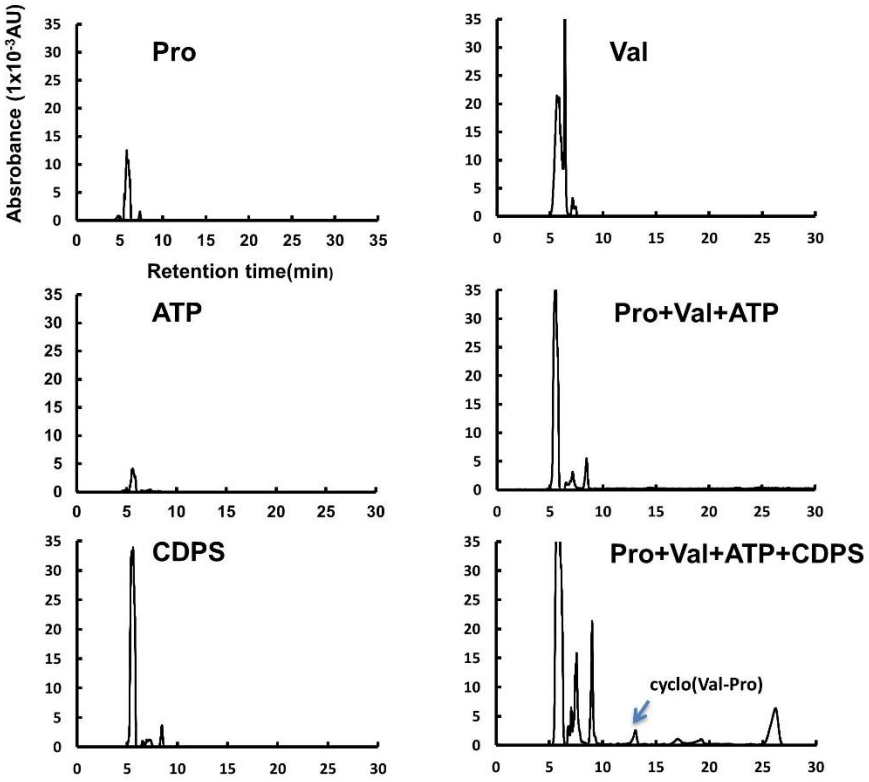


Fig. 23-4. HPLC analysis confirmed CDPS that catalyzed the synthesis of cyclo(Pro-Val) *in vitro*. L-Proline (upper left), L-Valine (left right), ATP (middle left) CDPS (lower left) peaks were shown in HPLC chromatogram. The reaction mixture of L-Proline, L-Valine and ATP presented no peaks after ten min (middle right). However, when CDPS was added, the reaction mixture of L-Proline, L-Valine, ATP and cyclic dipeptides showed a peak of new substance [which was confirmed as cyclo(Pro-Val)] later on around 13 min.

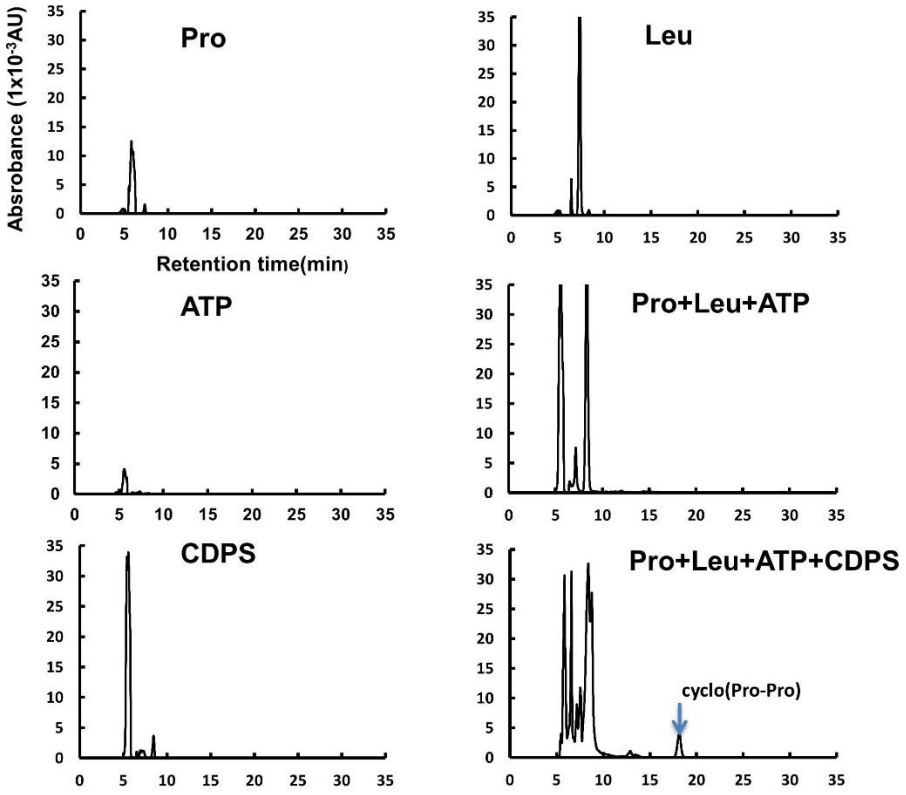


Fig. 23-5. HPLC analysis confirmed CDPS that catalyzed the synthesis of cyclo(Pro-Val) *in vitro*. L-Proline (upper left), L-Leucine (left right), ATP (middle left) CDPS (lower left) peaks were shown in HPLC chromatogram. The reaction mixture of L-Proline, L-Leucine and ATP presented no peaks after ten min (middle right). However, when CDPS was added in, the reaction mixture of L-Proline, L-Leucine, ATP and cyclic dipeptides showed a peak of new substance around 19 min.

Table 15-1 Mass analysis of products reacted with L-Proline and L-Phenylalanine

m/z of $[M+1^+$	m/z (%) of EI-MS	molecules
488	489.8(5.8), 488.9(17.8), 367.6(8.5), 337(3.5), 325(18.4), 249(6.4), 205(10.4), 163(40.1), 155.0 (2.45), 136(6.2),125.1 (2.2), 119(100), 105(65.8), 91.0 (33.9), 70.1 (5.5)	*cis*-cyclo(L-Phe-L-Pro), $(C_{14}H_{16}N_2O_2)$x2
294	295 (10), 267 (7.4), 239(17.8), 129 (34.2), 113.0 (31.9), 99 (37.3), 91 (32), 71 (83.3),	*cis*-cyclo(L-Phe-L-Phe), $C_{18}H_{18}N_2O_2$

Table 15-2 Mass analysis using EI and CI by GC-MS of products reacted with L-Proline and L-Lysine

m/z of $[M+1^+$	m/z (%) of EI-MS	Predicted molecules
225	256 (67.8), 241 (23.9), 213(38.7), 185 (42.2), 171 (27), 157 (20.9), 129(69.3), 115 (24.1), 111 (21.6), 97 (38.4), 85 (41.3), 83. (46.1), 73 (100), 61 (21.3) ,57(94.6)	*cis*-cyclo(L-Lys-L-pro), $C_{12}H_{24}N_4O_2$
256	256 (67.8), 241 (23.9), 213(38.7), 185 (42.2), 171 (27), 157 (20.9), 129(69.3), 115 (24.1), 111 (21.6), 97 (38.4), 85 (41.3), 83. (46.1), 73 (100), 61 (21.3) ,57(94.6)	*cis*-cyclo(L-Lys-L-lys), $C_{12}H_{24}N_4O_2$

Table 15-3 Mass analysis using EI and CI by GC-MS of products reacted with L-Proline and L-Valine

m/z of $[M+1^+$	m/z (%) of EI-MS	molecules
197.0	197 (7.1), 183. (6.6), 167.1 (6.9), 155.1 (11), 140.1 (5.0), 139.1 (6.0), 137.1 (17.1), 125.5 (7.8), 114.4 (4.9), 113.4 (16.5), 112.4 (19.4), 111.4 (24.6.), 110.4 (6.1), 107.3 (7.9), 99.4 (20.7), 98.3 (8.6), 97.1 (20.8), 96.3 (9.6), 84.1 (15.6), 83.1 (24.5), 82.1 (14.7), 73.2 (8.2), 72.3 (5.2), 71.1 (61.4), 70.3 (23.5), 69.1 (15.2), 68.1 (13.7)	*cis*-cyclo(L-Val-L-Pro), $C_{10}H_{16}N_2O_2$

Table 15-4 Mass analysis using EI and CI by GC-MS of products reacted with L-Proline and L-Serine

m/z of $[M+1^+$	m/z (%) of EI-MS	molecules
185.0	155.0 (12), 149.0 (12.7), 141.0 (8.6), 129.0 (13), 127.0 (15.4), 113 (10.8), 112.0 (11), 99.0 (12.5), 98.0 (8.1), 93.0 (10.6), 85.0 (29), 84.0 (9.9), 83.0 (27.1), 71.1 (31.8), 70.1 (18.9), 60.0 (14.4), 57.0 (73.1), 56.0 (10.9), 55.0 (30.2)	*cis*-cyclo(L-Ser-L-Pro), $C_8H_{12}N_2O_3$

Table 15-5 Mass analysis using EI and CI by GC-MS of products reacted with L-Proline and L-Leucine

m/z of $[M+1^+$	m/z (%) of EI-MS	molecules
195.0	195 (0.9), 184 (3.3), 178 (2.4), 155.1 (18.5), 154.1 (100.0), 153.1 (1.5), 138 (8.5), 129.1 (35.6), 111.1 (11.4), 100.1 (16.4), 90 (57), 84 (9.7), 70.1 (11.7) 61.1 (26.3), 60 (30),	*cis*-cyclo(L-Pro-L-Pro), $C_{10}H_{14}N_2O_2$

4. 3. 5. Molecular mass of CDPS in solution

To confirm the oligomeric state, purified CDPS protein (Fig. 25) was applied on Superose 12 10/300 GL column (GE Healthare) with molecular mass standards. A standard curve was generated by plotting the logarithm of melecular mass of standard proteins against their Kav, where kav= (Ve-Vo)/(Vt-Vo): Ve, elution volume; Vo, void volume; Vt, total ved volume. Kav of CDPS determined by using the same column was compared to the profile of protein standards; predicted molecular mass of CDPS is 99.18 kDa (Kav=0.5), indicating that CDPS exists as tetramer in solution (Fig. 25C).

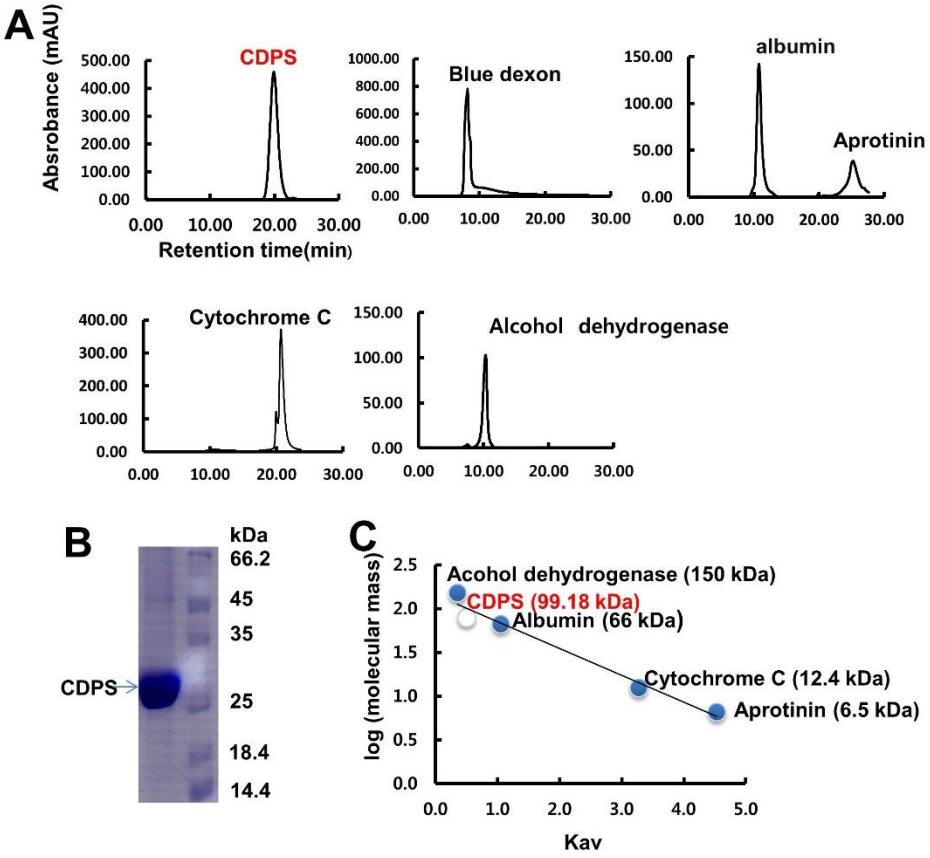


Fig. 25. Molecular mass was confirmed with superose 12 gel permeation column (GE Healthcare) for determining the oligomeric state. Superpose 12 gel permeation column used for determine the molecular weight with the standard contrast of aprotinin, cytochrome C, Albumin, and alcohol dehydrogenase (A). According the results of FPLC, the molecular of CDPS was calculated as 99.18 kDa (C). Purified CDPS was confirmed with 12 % SDS-PAGE as 25 kDa (B).

4. 3. 6. Structural determination of CDPS

To better understand the structure of CDPs, X-ray analysis was performed. Crystal native data sets and Se-Met substituted were collected at 100 K with area detector Systems Corporation (ADSC) Quantum 210 charge-coupled device area detector system at BL-7A at Pohang Light Source (PLS), South Korea. Diffraction data were processed and scaled with program *DENZO* and *SCALEPACK* (Otwinowski and Minor). Crystals belongs to the monoclinic space group, c2, with unit-cell parameters a=234.63, b=63.60, c=70.43 Å, and β=94.17. The solvent content of crystal was 39.3 % when the asymmetric unit is assumed to contain one molecule (Matthews 1968).

The four selenium sites were located (data not shown), and phase refinement was done by using the programs *SOLVE* and *RESOLVE* (Terwilliger 1999, Terwilliger and Berendzen 1999). Initial phasing and model building was with Se-Met CDPS MAD data set. Model building was done using the program COOT software (Emsley *et al.* 2010) and refined with REFMAC5 (Murshudov *et al.* 1997) from the CCP4 program suite (Collaborative Computational Project, 1994). Single crystal of CDPS was made in 50 mM $MgCl_2$, 100 mM HEPES pH 7.5 and 17 % PEG550 (Fig. 26) The native CDPS structure was refined to R_{work} of 39.3 and R_{free} of 11.6 in the resolution range of 5.0-2.08 Å. The refinement statistics of the native CDPS structure are summarized in Table 16.

Table 16 Data collection and refinement statistics of CDPS

	Native CDPS data
Wavelength (Å)	1
Temperature (K)	100
Resolution (Å)	0 2.08
Space group	**C2**
Unit cell parameters	a=234.63, b=63.60, c=70.43 , α=90, β=94.17, γ=90
Detector distance (mm)	300
Rotation range per image ( )	1
Exposure time per image (sec)	3
Total rotation range ( )	250
Completeness (%)[a]	80.9
Mean (*I*)	12.1
R_{sym} (%)[a,b]	39.3
Refinement	
Redundancy	2.8 (1.5)
Total reflections	**138,245**
R^c (R_{free}) (%)	11.6 (39.3)

a. The number in parentheses is for the outer shell.

b. $R_{sym}=\Sigma_h\Sigma_i/\Sigma_{h,i}-I_h/\Sigma_h\Sigma_i I_{h,i}$ where I_h is the mean intensity of the I observations of symmetry related reflections of h.

c. $R=\Sigma/F_o-F_c/\Sigma F_O$, where $F_o=F_p$, and F_c is the calculated protein structure factor from the atomic model. R_{free} was calculated with 10 % of the reflections.

d. $R=\Sigma/F_o-F_c/\Sigma F_O$, where $F_o=F_p$, and F_c is the calculated protein structure factor from the atomic model. R_{free} was calculated with 10 % of the reflections.

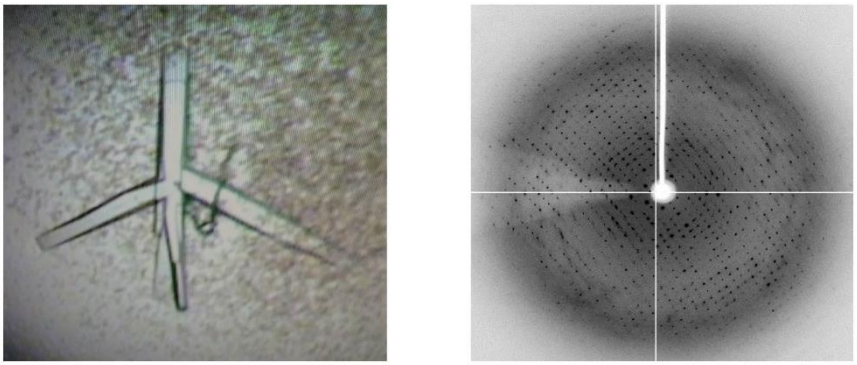


Fig. 26. Crystals and diffraction images of CDPS.

A: The crystals of CDPS.

B: The diffraction image of CDPS of X-ray radiation.

4. 3. 7. Overall Structural of Recombined CDPS

According to the ribbon model of the CDPS as shown as Figure 27, CDPS was composed as a tetramer structure with 4 identical subunits. In terms of the secondary structure, the subunit has 3 main layers of alpha/beta/alpha (Fig. 28). It contains a mixed bate sheet of 6 strands with one strand existing as an anti-parallel strand to the rest. Totally 7 alpha helix and 5 3_{10} helix surround the beta sheet layer. Four subunits polymerized together symmetrically and form a hollow space in the center (Fig. 29). The similar binding site were searched through PDBj database (http://pdbj.org/giraf/). Interestingly the result reveal the sequence of CDPS are comparable with human phosphoglycerate mutase 1 (PGM1), a member of alkaline phosphatase superfamily, with a similarity score of 74 % (Fig. 30). Noticeably, except PGM enzyme of yeast which is a homotetramer of mass 110,000 kDa, PGM enzymes are usually homodimer molecular. Thus, we confirmed CDPS isolated from lactic acid bacterial is a novel member of alkaline phosphatase superfamily.

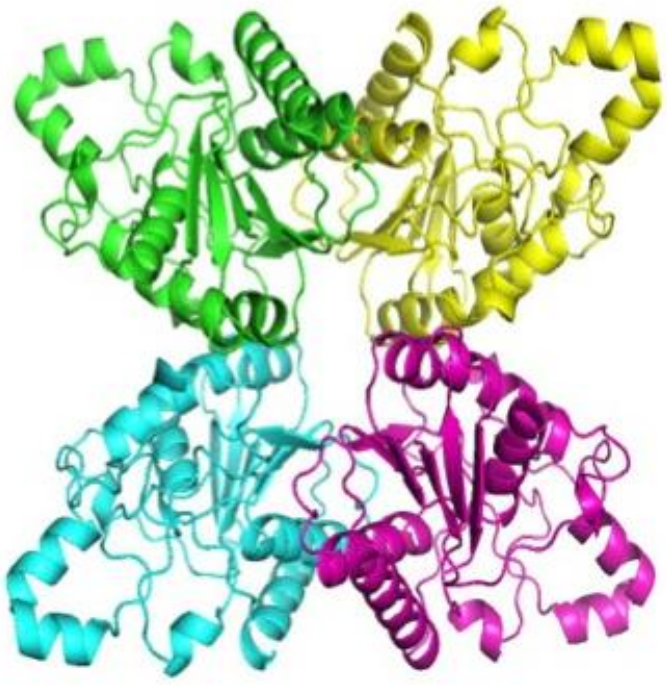
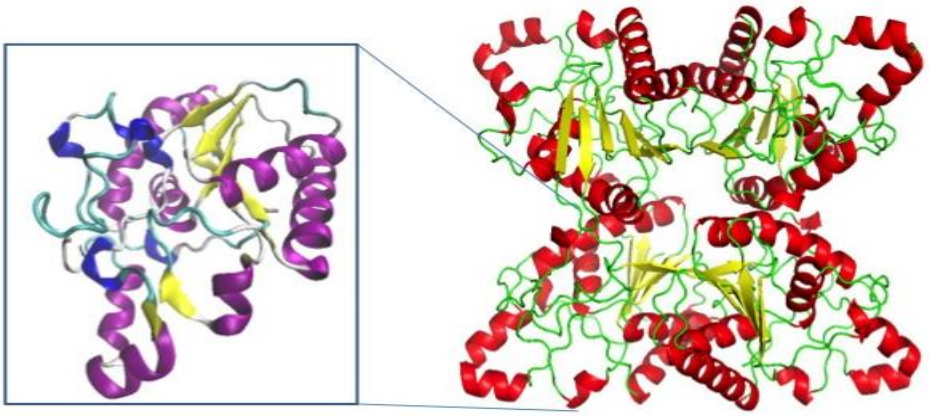


Fig. 27. The homotetramer structure of CDPS. As shown in the new cartoon model, CDPS was constructed by four identical subunits.



Subunit A

Fig. 28. The secondary structure of CDPS subunit. For a better view, the subunit A were colored depended on the secondary structure (left panel). Each of the identical subunits contains 6 beta sheets (yellow), surrounded by 7 alpha helix (purple) and 5 3_{10} helix (blue). Thus, each subunit has 3 layer of alpha/beta/alpha structure.

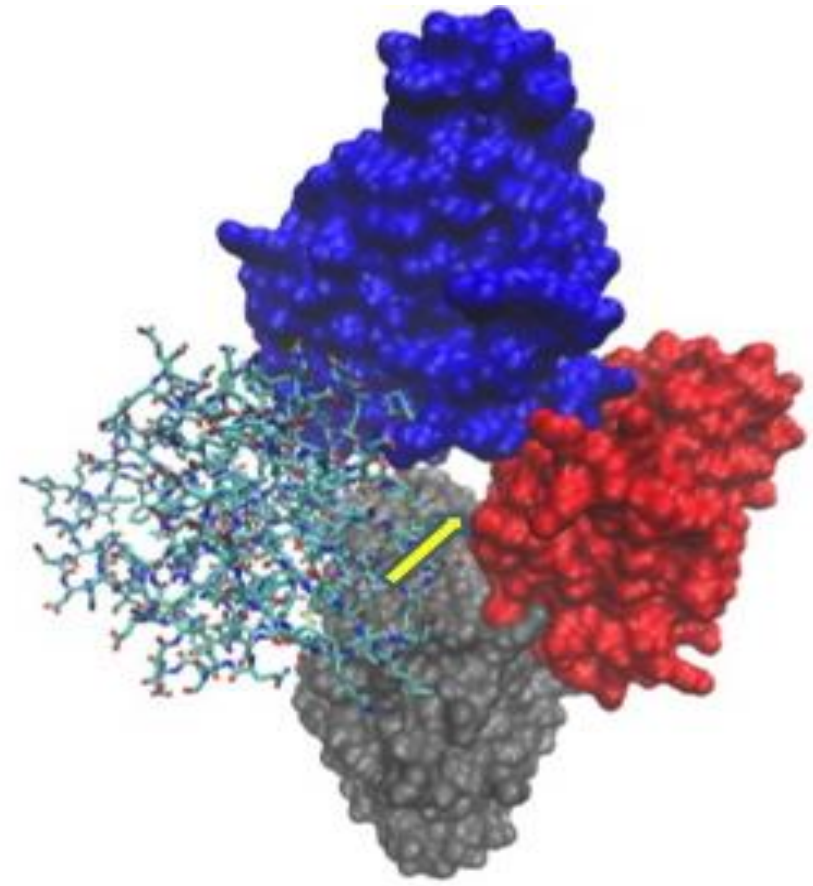


Fig. 29. A hollow space centered in the structure of CDPS (Arrow). A hollow space centered in the structure of CDPS (indicated by yellow arrow).

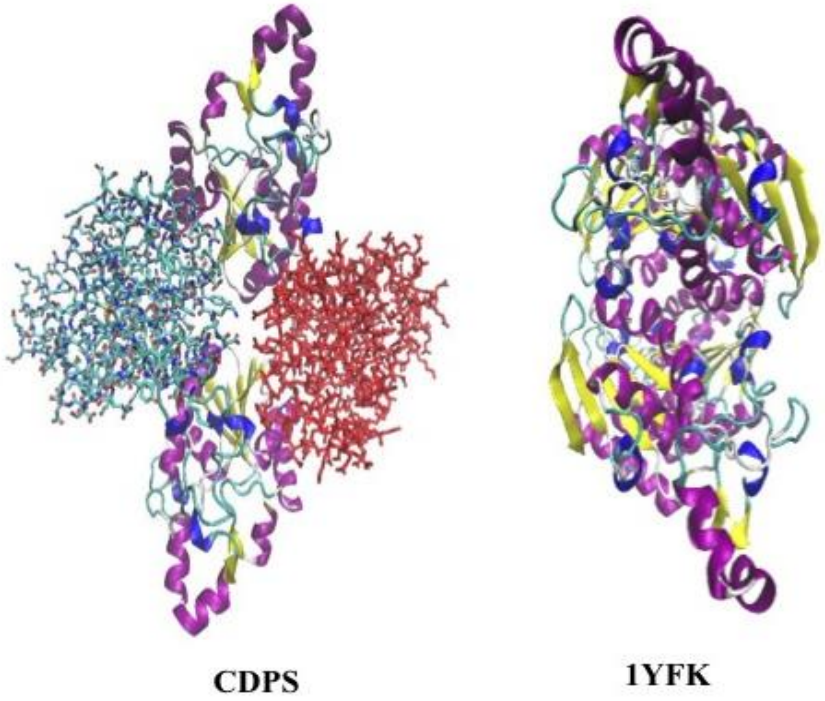


Fig. 30. Comparation of cyclic dipeptide synthetase and PGM1 (PDBID: 1YFK). Through PDBj data base, it is confirmed cyclic dipeptide synthetase contains the similar combining site with human PGM1.

4. 4. Discussion

Unwiring the structure and function of CDPS is pivotal for revealing the physiological processing of CDPs synthesis in microorganism as well as inventing *in vitro* bioreactor for producing functional CDPs. In this study, we identified a kind of new CDPS protein which displayed the capability of synthesis antibiotic CDPs, especially proline based CDPs. When transferring this gene into *E. coli*, functional CDPS could be obtained. Moreover, with HPLC confirmation, we successfully defined an *in vitro* reactions system which consisted with CDPS, amino acid, ATP in PBS buffer solutions. We figured out that, in 37 ℃ and ATP supplement, CDPS could synthesize the target CDPs. However, compare with the *in vivo* system, the synthesizing efficacy was quite low. This may indicate that for higher yield of CDPs, other auxiliary molecular may be needed besides more optimal physiochemical conditions should be defined. Moreover, we noticed CDPS especially catalyze the synthesis of CDPs with proline based DZK ring structure.

We further analyzed the 3 dimensional structure of CDPS by X-ray crystallographic analysis (data not shown). We are now working for identify the catalytic site in order to reveal the synthesis process in more detail.

As a conclusion, it was firstly reported the CDPS like function of an unnamed protein (gi311821850), and successfully development the *in vitro* CDPs synthesis conditions based on the newly found CDPS protein, CDPS. These studies will contribute to producing CDPs *in vitro* through biosynthesis pathway and to discover physiology mechanisms about functional CDPs synthesis pathway in microorganism.

CHAPTER V.

Genernal conclution

Studies elucidate CDPs produced by LAB were important for their probiotic effect on human health. In this study, we firstly extract CDPs with MC. The filtrates were separated on anion exchangers by applying them to a combination of the weakly basic or strongly basic anion resins Amberlite IRA-67 and Purolite A420S, respectively. Organic acids and sugars, were removed using three types of anion exchange chromatography. The resultant eluents were extracted with MC as a secondary purification step. The MC-extracted samples were reseparated by HPLC and a combined fraction including F1 to F17 was obtained. Additionally, owing to the repeated MC extraction during the purification steps, the eluents contained pure CDPs. All of the CDPs with activity against bacteria, fungi and viruses, including cis-cyclo(L-Val- L-Pro) (F7), cis-cyclo(L-Leu- L-Pro) (F13) and cis-cyclo(L-Phe- L-Pro) (F17), significantly increased in the stationary phase of cell growth. This observation coincided with the increase in antifungal fractions, including cis-cyclo(L-Tyr- L-Pro) (F6), cis-cyclo(L-Val- L-Pro) (F7), cis-cyclo(L-Ser- L-Pro) (F9), cis-cyclo(L-Leu- L-Pro) (F12) and cis-cyclo(L-Phe- L-Pro) (F17). HPLC profile of the total pool of CDPs naturally produced by the isolates showed an identical pattern.

Although many studies investigated the anticancer activities of LAB, its effect on non-GI tract cancer has been rarely studied. Since the absorption of CDPs was proved previously and the stability of its special chemical structure, we expect LAB are capable of inhibiting the growth of breast cancer. In this study, the cancer prevention effect of *Lb. plantarum* LBP-K10 were confirmed first on in situ breast cancer model in mice by oral administration of cell free CF-K10. CF-K10 may induced apoptosis in breast cancer

cell line MDA-MB-231 by activation apoptosis pathways. The expression of caspase-3 and cytosolic cytochrome C were elevated when the cells treated with MC-K10. Ac-DEVD-CHO, an inhibitor of caspase-3 reversed apoptosis effect of MC-K10 treatment. These results indicate MC-K10 isolated from CF-K10 could induce breast cancer cell apoptosis through caspase-3 pathway. The sphere formation ability and partition of CD133+ cells were decrease dramatically in MC-K10 treated group thought the decease of sphere formation ability and CD133+ cell partition were milder in cyclo(Phe-Pro) administration group. Compare with cyclo(Phe-Pro) treatment, the expression of Oct4 was also dramatically down regulated in MC-K10 treated group. These results indicated MC-K10 could induce the stemness and self-renew ability of breast cancer stem cells. These results provided new insight for the probiotic active of LAB, and also contribute to develop new strategies for breast cancer privation.

These studies revealed as a nature product form LAB, CDPs has great potential in antibiotic or even anti cancer activites. The special and rigid structure of CDPs were also attracted great attention for developing new drugs. Therefore, we next tried to reveal the machanism of CDPs synthesis in *Lb. plantarum* LBP-K10, so that we can increase the level of CDPs content in *Lb. plantarum* LBP-K10 or develop methods for synsthesis CDPs *in vitro*. In this study, a new CDPS was identified. When transfecting this gene into *E. coli*, CDPs production were upregulated, especially CDPs with L-proline based diketopiperazine ring structure. Moreover, confirmed by HPLC, cyclic dipeptides were also successfully obtained in an *in vitro* reaction system which consisted with recombined CDPS, amino acid, ATP in buffer solutions. Several pH conditions were

tested in each amino acid combinations (L-Proline and L-Lysine; L-Proline and L-Phenylalanine; L-Proline and L-Valine; L-Proline and L-Serine; L-Proline and L-Leucine) and the amount of the cyclic dipeptides producing in the *in vitro* condition was measured by HPLC. However, compare with the *in vivo* system, the synthesizing efficacy was quite low. It may indicate that for higher yield of cyclic dipeptides, other auxiliary molecular may be needed, or more optimal physiochemical conditions should be defined. In order to better understand that the mechanism of cyclic dipeptides synthesis in molecular level, we analyzed the 3 dimensional structure of CDPS by X-ray crystallographic analysis. CDPS was composed as a tetramer structure with 4 identical subunit showed in the ribbon model. Thus, it was confirmed that CDPS isolated from *Lb. plantarum* LBP-K10 was a novel member of alkaline phosphatase superfamily. We are now further working for identify the catalytic site in order to reveal the synthesis process in more detail.

Above of all, antimicrobial CDPs produced by *Lb. plantarum* LBP-K10 were purified and characterized. Totally eight antibiotic components were identified. Also, a massive purification method was proposed. CDPs complex produced by *Lb. plantarum* LBP-K10 are capable of prevention breast cancer by targeting cancer stem cells. The CDPS like function of an unnamed protein (gi311821850) was firstly reported and it was successfully used as developed the *in vitro* cyclic dipeptides synthesis conditions. This study provided new insight for understanding the probiotic effect and applications of LAB. It was also contribute for developing new strategies for generating CDPs more effectively and thus benefit to anticancer antimicrobiotic drug design based on structure.

CHAPTER VI.

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국문초록

유산균 (LAB)은 인간의 건강을위한 프로 바이오 틱 미생물로 인정 받고 있다. LAB 에 의해 생성 된 사이클릭 디 펩티드 (CDP 가)는 자신의 항생제 활동에 중요하다. CDP 가 디케 토피 페라 (diketopiperazine) 구조의 여러 종류의 분자의 그룹이고, 약물 설계를 위한 매력적인 골격을 역임한다. 이러한 항생 활성 물질로서. 유산균 배양액 내의 CDP 성분이 보고된 바가 미미하며 한 균주에서 모든 CDP 를 규명한 보고가 없었다. 따라서 본 연구는 락토바실러스 플라타룸 LBP-K10 (*Lactobacillus plantarum* LBP-K10) 에서 생산되는 항생 CDP 를 복합체 형태로 분리하고 각각의 CDP 를 순수분리하여 이를 확인하여 항생 활성에 대하여 관찰하였다. 모두 일곱 CDP 가 가스 크로마토 그래피 - 질량 분석을 통해 확인 하였다. 음이온 교환 수지인 Amberlite IRA-67 와 Purolite A420S 를 사용하여 column chromatographs 를 통해 배양액 여과액에 있는 모든 CDP 를 대량 분리하는 방법을 제공하였다. 순수분리한 싸이클릭 디펩타이드 복합체는 다제내성균,

인간 및 식물 특이성을 가지는 병원성 진균과 인플루엔자 바이러스에 대해서 단일 싸이클릭 디펩티드와 비교하여 볼 때 수십배의 활성 상승 효과를 보였다.

최근 연구는 가지 메커니즘에 의해 암 진행을 억제 할 수도있는 유산균 또는 생산물을 밝혔다. 그러나 대부분의 연구는 위장관 관련 암, 특히 결장암에 초점을 맞춘다. 비 위장관 암에 관련 유산균 또는 생산물의 효과가 거의 관찰되지 않았다. CDP 가 효율적으로 흡수 입증하기 때문이라는 연구가 있어, 유산균이 비 - 위장관 암의 일종인 유방암에 대한 암 예방 효과조사 하였다. LBP-K10 배양 여액에서 주로 CDP 를 포함한 메틸렌 클로라이드 추출 분획 (MC-K10)를 동정하였다. 본 연구에서는, 경구 적용 CF-K10 동물 모델에서 유방암 성장의 진행을 억제하는 것으로 처음으로 확인되었다. 또한 CF-K10 및 MC-K10 는 MDA-MB-231 세포에서 세포 사멸을 유도 할 수 있음을 발견했다. 또한, 암 줄기 세포의 stemness 와 자가 갱신을 손상시킬 수 MC-K10 및 시클로 페닐알라닌 (Phe-Pro)를 입증도

하였고. 이연구들은 새로운 LAB 및 제품의 프로바이오틱스 활성에 대한 통찰력과 유방암 예방 전략의 개발을 위한 힌트를 제공을 제공한다.

인간의 히스에 CDP 가의 중요성과 이점은 CDP 생합성 과정을 조사하기를 격려했다. 닌히 드린 활성 염색에 의해, 싸이클릭딥펩티드 합성 효소 (CDPS)에 의존 경로를 통해 *Lb. plantarum* LBP-K10 에서 활성물질인 CDP 를 합성함을 확인할 수 있었다. *Lb. plantarum* LBP-K10 에서 순수분리된 CDPS 는 2D LC MS/MS 를 사용하여 분자량과 단백질의 이차 구조를 예측할 수 있었다. 락토바실러스 플란타룸 LBP-K10 의 CDPS 는 분자량이 26.1 kDa 이며 (gi311821850) 690 bp 의 open reading frame 을 유전자 서열을 가짐을 확인하였다. 흥미롭게도, 이 CDPS 유전자를 대장균에 형질전환을 수행한 결과, 다양한 형태의 proline 을 가지는 CDP 의 발현이 증가함을 관찰하였다. 이와 동시에 대장균에 형질전화한 CDPS 의 발현은 SDS-PAGE 에서 CDP 를 합성하는 최적의 *in vitro* 조건도 동시에 확인할 수 있었다. 또한 분자 수준에서 CDP 를 합성기전을 이해하기 위해서 효소를 x-

선 회절 분석을 수행하였다. 결정은 2.6 Å 의 해상도로 분석하였고 C2 space group 이며 리본모델에서 CDPS 가 4 개 동일한 서브유닛과 사양구조로 보였다. 본 연구는 처음으로 새로운 CDPS 를 동정하며 CDPS 가 CDPs 를 생성하는 체외(*in vitro*)조건을 확인하였다. 본 연구는 체외에서 자연 항생제 CDP 를 생합성 하는 메커니즘과 과정을 이해하는 데 도움이 될 것이다.

무엇보다도, 이러한 연구들에서 *Lb. plantarum* LPB-K10 에 전체는 CDPS 프로파일 식별하였으며, 유산균과 유산균이 생산한 CDP 들의 암 예방 효과를 입증하였고, CDPS 를 처음으로 동정하였다. 이 연구는 CDP 가 생합성 메커니즘을 이해하기위한 프로 바이오 틱 유산균과 그의 혜택에 새로운 통찰력을 제공하였다.

주요어: 사이클릭 디 펩티드, 싸이클릭딥펩티드 합성 효소, 락토바실러스 플라타룸 LBP-K10, 유방암, 암 예방, 암 줄기 세포, 다제내성균, 병원성 진균, 인플루엔자 바이러스, 시클로 페닐알라닌